Mycobacterium ulcerans disease and treatment: a histopathological perspective

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Prof. Dr. Martin Spiess
Dekan

Dedicated to my parents
and my grandfather Werner Reim

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Summary

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a chronic necrotizing skin disease with the highest prevalence in West-African countries. The current WHO treatment recommendation is a combination of the two antibiotics rifampicin, given orally and streptomycin, requiring daily injections. Although antibiotic treatment reduced recurrence rates below 2% and circumvents surgical excision in part of the patients, toxic side effects, immunopathological adverse events and other wound healing disorders may occur. In this thesis we conducted detailed histopathological studies to better characterize responses to antibiotic treatment and the nature of paradoxical reactions.

In the case of non-ulcerative plaque lesions, we observed in half of all patients either an enlargement or ulceration of lesions during antibiotic therapy. Histopathological analysis after completion of antibiotic treatment revealed the persistence of extensive necrotic areas besides hallmarks of successfully treated BU lesions, like infiltration, granuloma formation and loss of solid staining of the mycobacteria. Where removal of the necrotic tissue by the immune system is not efficient enough, lesions are ulcerating, leading to the discharge of necrotic tissue. Based on the clinical and histopathological data it is suggested to support healing of such plaque lesions by surgical débridement. While our data demonstrate that the antibiotic therapy efficiently destroys *M. ulcerans* infection foci, they also indicate that proper wound management during and after chemotherapy is for advanced BU lesions as important as the antibiotic treatment itself.

Secondary lesions may occur at distant body sites during and after chemotherapy. Our analysis of such secondary lesions revealed hallmarks of inactive *M. ulcerans* infection. Emergence of secondary lesions during antibiotic treatment may thus be the result of immune-mediated paradoxical reactions driven by mycobacterial antigens and immunostimulators at sites of clinically inconspicuous infection foci. These lesions may, however, also have arisen from new infections or mycobacteria that have survived chemotherapy. In this case our histopathological findings would indicate that after priming during the successful treatment of the initial lesion, the immune system is capable of eliminating new infection foci. Our results demonstrate

that no additional antibiotic treatment is necessary, when such secondary lesions appear.

To reduce the danger of toxic side effects and to obviate the need for daily injections, an entirely oral treatment without streptomycin would be preferable. In the framework of this thesis we analyzed tissue samples from a first clinical treatment trial using clarithromycin instead of streptomycin. Histopathological findings and efficacy were comparable to the current treatment, leading to the planning of a WHO-sponsored large multi-center trial comparing the efficacy of eight weeks treatment with clarithromycin and rifampicin versus streptomycin and rifampicin.

To gain deeper insights into the histopathological changes in the very early phase of *M. ulcerans* infection, we conducted in parallel to the analysis of clinical samples, longitudinal histopathological studies in experimentally infected mice. Many features found in the mouse model, such as an early wave of infiltrating neutrophils, as well as the formation of B-cell clusters and the loss of solid Ziehl-Neelsen staining of mycobacteria during chemotherapy, have correlates in human BU lesions. The mouse infection model thus appears to be suitable for the preclinical evaluation of new drug treatments and of candidate vaccines.

Taken together the results described in this thesis demonstrate that histopathology is an important tool to strengthen diagnosis of BU, to evaluate new treatment regimens and to come to a better understanding of paradoxical reactions emerging during antibiotic treatment of BU.

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Zusammenfassung

Das Buruli-Ulkus (BU) ist eine nekrotisierende Hauterkrankung, die von *Mycobacterium ulcerans* hervorgerufen wird und vor allem in den tropischen Regionen West-Afrikas auftritt. Gegenwärtig empfiehlt die Weltgesundheits-organisation (WHO) eine systemische Chemotherapie mit dem oral verabreichten Antibiotikum Rifampicin kombiniert mit einer täglichen Injektion von Streptomycin. Nach dieser Therapie treten bei weniger als 2% der Patienten Rückfälle auf und eine chirurgische Zusatzbehandlung der Läsion ist bei vielen Patienten nicht erforderlich. Allerdings können toxische Nebenwirkungen sowie immunpathologische Reaktionen und andere Wundheilungsstörungen auftreten. In der vorliegenden Arbeit wurden detaillierte histopathologische Studien durchgeführt, um die therapeutische Wirksamkeit der Chemotherapie des BU besser zu verstehen und die Natur von paradoxen Reaktionen zu charakterisieren.

Bei vielen Patienten mit zunächst geschlossenen BU Plaques kommt es während der Chemotherapie zur Ulzeration. Unsere histopathologischen Analysen zeigten, dass auch nach Beendigung der vollständigen Antibiotika-Behandlung noch grosse nekrotische Gewebebereiche zurückbleiben können. Daneben sind andererseits auch typische Merkmale einer behandelten BU-Läsion wie Infiltration, Granulom-Bildung und Verlust der vollständigen Anfärbbarkeit der Mycobakterien zu beobachten. Unvollständige Resorption des nekrotischen Gewebes durch das Immunsystem führt jedoch häufig zur Ulzeration und Abstossung des nekrotischen Gewebes. Basierend auf unseren histopathologischen und klinischen Daten erscheint es angezeigt, die vollständige Ausheilung von Plaques durch chirurgisches Debridement zu beschleunigen. Die Befunde zeigen, dass *M. ulcerans* Bakterien durch die Chemotherapie mit Rifampicin und Streptomycin zwar effektiv abgetötet werden, dass aber vor allem im fortgeschrittenen Stadium eine gute Wundversorgung für den Heilungserfolg beim BU ebenso wichtig ist.

Bei einem Teil der BU Patienten entwickeln sich während oder nach Abschluss der Chemotherapie sekundäre Läsionen. Bei der Analyse solcher Läsionen haben wir charakteristische Merkmale einer inaktiven *M. ulcerans* Infektion gefunden. Der Auslöser für die Entwicklung von Sekundärläsionen könnte daher eine paradoxe Immunreaktion gegen noch im Gewebe vorhandene mykobakterielle Antigene bei

klinisch zuvor nicht auffälligen Infektionsherden sein. Möglich wäre allerdings auch, dass sich nach der Chemotherapie durch überlebende Mycobakterien oder durch Neuinfektion neue Läsionen gebildet haben. In diesem Fall würden unsere Ergebnisse darauf hindeuten, dass die neuen Infektionsherde durch eine während der Erstinfektion gebildete protektive Immunantwort zerstört worden sind. Insgesamt zeigen unsere Untersuchungen, dass eine erneute Antibiotikagabe beim Auftreten von sekundären Läsionen nicht erforderlich ist.

Zur Reduzierung der Gefahr toxischer Nebenwirkungen und zur Vermeidung der täglich erforderlichen Injektionen, wäre eine vollständig orale Chemotherapie ohne Streptomycin anstrebenswert. Bei einer ersten klinischen Studie, bei der Streptomycin durch Clarithromycin ersetzt wurde, waren Wirksamkeit und histopathologische Beobachtungen vergleichbar zu den Ergebnissen mit der gegenwärtigen Standardtherapie. Basierend auf diesen Ergebnissen plant die Weltgesundheitsorganisation eine multizentrische klinische Studie, bei der 8 Wochen Rifampicin/Clarithromycin mit 8 Wochen Rifampicin/Streptomycin verglichen werden soll.

Um einen tieferen Einblick in die frühen Phasen der *M. ulcerans* Infektion zu bekommen, wurden longitudinale histopathologische Untersuchungen mit experimentell infizierten Mäusen durchgeführt. Viele histopathologische Befunde, wie das Auftreten einer ersten Infiltration mit Neutrophilen in der Frühphase der Infektion und die Entwicklung von B-Zell-Anhäufungen und der Verlust der gleichmässigen Anfärbbarkeit der Mycobakterien während der Chemotherapie haben Entsprechungen bei der BU Erkrankung beim Menschen. Das Mausmodell scheint daher geeignet zu sein, neue Therapien und Kandidaten-Impfstoffe präklinisch zu testen.

Insgesamt zeigen unsere Ergebnisse, dass die Histopathologie nicht nur ein sehr aussagekräftiges Diagnosewerkzeug ist, sondern dass sie auch einen wichtigen Beitrag zur Beurteilung neuer Behandlungsmethoden und zur Erforschung paradoxer Reaktion während der Antibiotika Behandlung leisten kann.

Abbreviations

AFB	Acid Fast Bacilli
BALB/c	mouse strain
BCG	Bacillus Calmette-Guèrin
BU	Buruli ulcer
CFU	Colony forming units
CTUB	Centre de dépistage et de Traitement de l'Ulcere de Buruli
DCs	Dendritic cells
HE	Haematoxylin- Eosin staining
IFN	Interferon
Ig	Immunglobulin
IHC	Immunohistochemistry
IS	Insertion sequence
mAb	monoclonal antibody
MU	Mycobacterium ulcerans
PCR	polymerase chain reaction
qPCR	quantitative PCR
PFA	Paraformaldehyde
PPD	Purified Protein Derivative
R/S	Rifampicin and Streptomycin
RIF/STR	Rifampicin and Streptomycin
RIF/CLR	Rifampicin and Clarithromycin
TB	Tuberculosis
WHO	World Health organization
ZN	Ziehl-Neelsen staining

Chapter 1

Introduction

The neglected tropical necrotizing skin disease Buruli ulcer (BU) is the third most common mycobacterial disease after tuberculosis and leprosy and is caused by an infection with *Mycobacterium ulcerans*[1]. The main burden falls on children below the age of 15 in rural West-African countries but in general people of all ages, races and socioeconomic classes are susceptible [2].

1.1 History and Epidemiology

The disease was first discovered and described as “the presence of large ulcers” in 1897 by Sir Robert Cook in Uganda and later by Kleinschmidt in northeast Congo during the 1920s [3]. The definitive description of BU and the isolation of the causative agent *Mycobacterium ulcerans* was first published in 1948 by Mac Callum and colleagues. They described six Australian patients with unusual skin lesions caused by a mycobacterium which only grew at a lower temperature than usually used for cultivating *Mycobacterium tuberculosis* [4].

Between the 1960s and 1980, several African countries reported about focal areas endemic for BU including Congo, Uganda, Gabon, Nigeria, Cameroon and Ghana [5]. Because the first reported cases came from the Buruli region of Uganda near Lake Kyoga the Uganda Buruli group introduced the name “Buruli”[6]. Dramatic increases in the reported incidence of BU have been reported since 1980, especially in West-African countries, and more than 30 countries worldwide are affected today (Figure 1) [7,8]. In some countries reported detection rates were higher than those for leprosy and tuberculosis [9]. New foci are still today constantly reported. Some of these may have developed because of man-made environmental alterations (dammed lakes, water irrigation systems, deforestation) [10]. In remote areas of developing countries case detection and surveillance is difficult which might lead to an under-reporting.

In 1998, the Yamoussoukro declaration on Buruli ulcer recognized BU as a re-emerging infectious disease with a significant public health impact and called on the international community to support control and research efforts[11]. Last reports of the WHO indicated that more than 5000 people annually are diagnosed with BU [7].

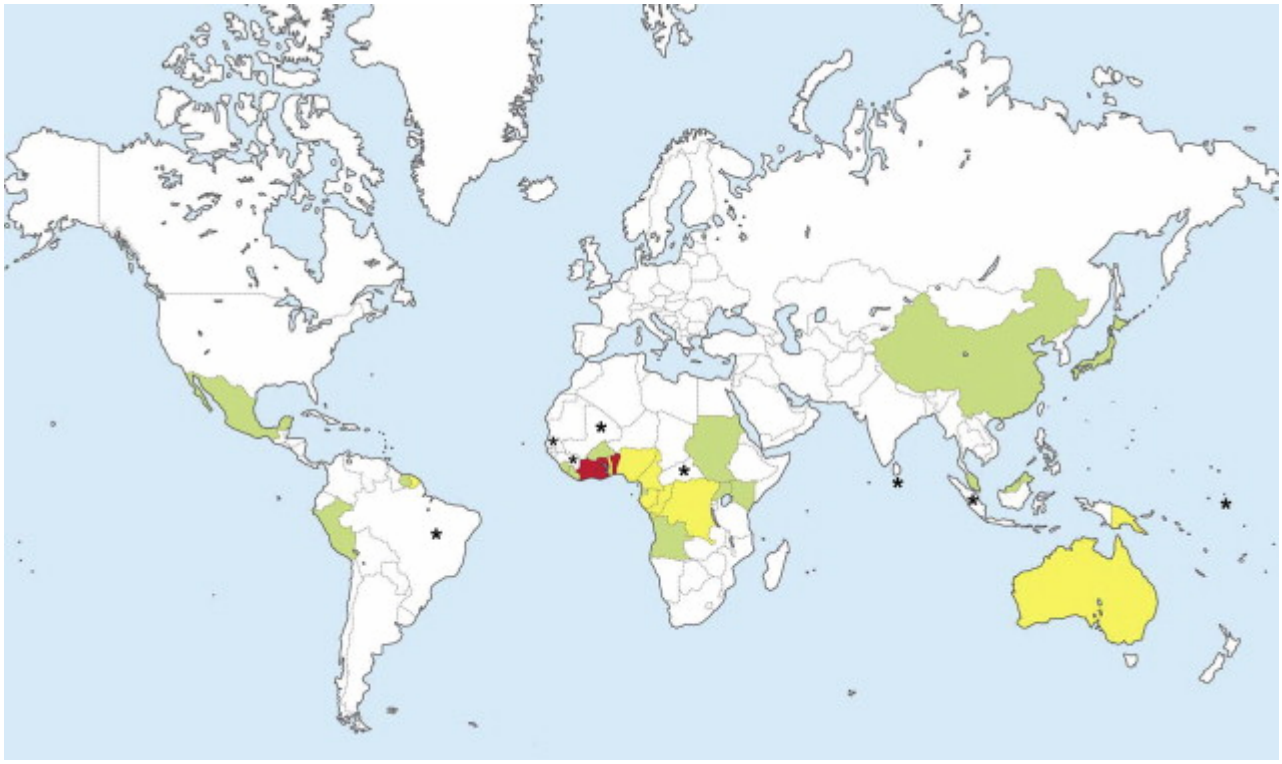

Figure 1: Distribution of BU by country, 2010. Relative endemicity is denoted as high (red), moderate (yellow), and low (green). Asterisks denote countries with suspected cases. (Source: Walsh DS et al, Dermatol Clin. 2011)

1.2 Causative organism

Traditionally, *M. ulcerans* was thought to belong to the large group of environmental or so called "occasional pathogens" which can cause disease in humans and animals. Usually those bacteria have an environmental reservoir and infect their host accidentally [12]. However, for *M. ulcerans* an environmental reservoir has not been identified so far. Isolation from the environment is a major challenge, since *M. ulcerans* is a slow growing bacterium which grows best at 30°C on Löwenstein-Jensen medium. Primary cultures from BU lesions are difficult to obtain often taking several months to grow [13].

1.2.1 Genome and genetic diversity of M. ulcerans

Phylogenetic studies indicate that *M. ulcerans* has evolved from a common *M. marinum* progenitor. Despite their contrasting phenotypes, *M. ulcerans* and *M. marinum* have almost identical genome sequences. Acquisition of a virulence

plasmid, incorporation of multiple copies of two insertion sequences (IS2404 and IS2606) into the genome and genome reduction by gene deletion are hallmarks of the development of *M. ulcerans* [14-17]. *M. ulcerans* strains can be either assigned to the ancestral lineage (strains from Asia and South America) which is closer related to *M. marinum* or to the classical lineage (strains from Africa and Australia) which seem to be more virulent [18]. *M. ulcerans* strains from Africa reveal a high clonality making genetic fingerprinting and molecular epidemiological analysis extremely demanding.

1.2.2 Mycolactone

Much of the pathology and virulence of *M. ulcerans* seems to be attributed to the polyketide macrolide toxin mycolactone which is the major component of the acetone-soluble lipid extract of an *M. ulcerans* culture [19]. Different forms, named Mycolactone A to F, are found in *M. ulcerans* strains from different countries or in other mycolactone producing mycobacteria. Extracted as well as synthetically produced mycolactone exhibits cytotoxic and immunosuppressive features *in vitro* as well as in vivo. When added to fibroblasts and macrophages, effects on the cytoskeleton, cell growth arrest, necrosis and apoptosis are observed. Injection of purified mycolactone into the skin of guinea pigs induces ulceration comparable to BU lesions [20]. Mycolactone extracted from human infected tissue shows chemical and biological properties of mycolactone A/B [21]. It is assumed that mycolactone is the cause of the limited immune response inside the necrotic core of BU lesions, although large clusters of extracellular bacteria and extensive tissue damage are observed [20]. In particular, T-cell responses seem to be suppressed [22]. In mice, destruction of nerves by mycolactone can be observed and might explain the painlessness of BU lesions in humans [23,24].

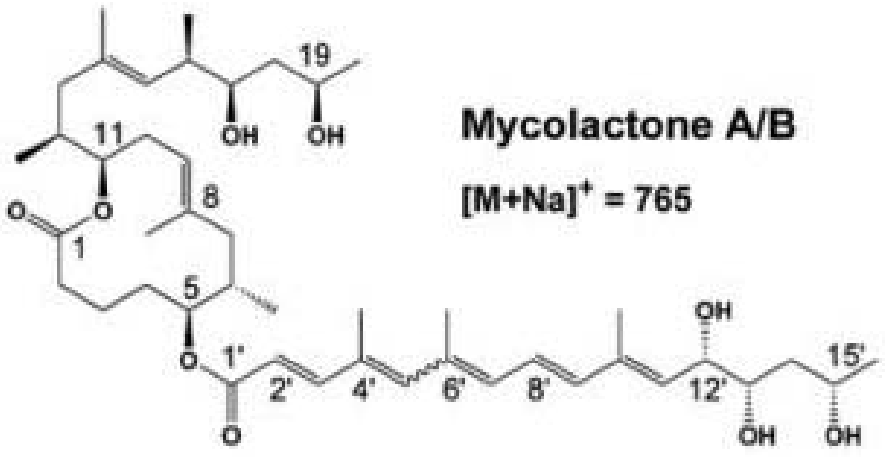

Figure 2: Mycolactone A/B. (Source: Stinear et al, Natural Product Reports, 2008)

1.3 Reservoir and Transmission

Both, transmission as well as the environmental reservoir of *M. ulcerans* still remain a mystery [25]. Endemic foci are associated with slow flowing or stagnant water with hot and humid climate (except for the temperate region of southern Australia) indicating water as a risk factor. Also men made environmental modifications such as dams, irrigation systems or deforestation are thought to increase the risk of acquiring BU [1,10,26,27]. Trauma is probably the most frequent means by which *M. ulcerans* might be introduced into the skin from environmental sources [27,28].

M. ulcerans was first detected in the environment with the help of the Polymerase Chain Reaction (PCR) targeting a *M. ulcerans* specific Insertion sequence element (IS2404) [29,30]. DNA of *M. ulcerans* was also found in aquatic insects obtained from BU endemic areas leading to the hypothesis that bites of water insects might transmit the disease. Experimentally infected water insects, *Naucoridae*, were able to transmit *M. ulcerans* to laboratory mice leading to the formation of typical ulcers [31,32]. Additionally, *M. ulcerans* DNA was detected in water, biofilms, plants and snails [33-36]. Until today only one culture of an environmental *M. ulcerans* strain could be isolated from a water strider supporting the concept that the agent of BU is a human pathogen with an environmental niche [37].

Studies in Australia revealed a strong correlation between the incidence of BU and the incidence of vector borne infections (Ross River Virus). Mosquitoes captured during an outbreak of BU in a highly endemic area in southern Australia (Point Lonesdale) identified 4.3/1000 IS2404 positive mosquitoes [38]. Recently Fyfe et al

discovered that up to 38% of possums, small terrestrial marsupials, captured at Point Lonesdale had laboratory confirmed *M. ulcerans* skin lesions and/or PCR positive faeces [25,39].

1.4 Pathogenesis

1.4.1 Clinical presentation

BU is characterized by a spectrum of clinical forms, either as a non-ulcerative lesion or as an open ulcer. A papule, nodule or oedema is usually a sign of an early BU lesion which can progress to ulcers with undermined edges and cotton wool-like discharge. Most ulcers are painless unless secondary infections with other bacteria occur [7,40]. Any part of the body can be affected but lesions mostly occur at the extremities [41]. The incubation time is assumed to range between a few weeks up to years. Patients usually present late at the hospital when large ulcers are already established, leading to long hospital stays, disabling contractures, scarring or even amputation [9,42]. Approximately 10% of the patients develop bone involvement subjacent to skin lesions or metastatic osteomyelitis from lymphohematogenous spread [7,43].

Figure 3: Various stages of BU (Source: WHO)

1.4.2 Histopathology

Typical histopathological features found in tissue sections from untreated BU patients include coagulative subcutaneous necrosis of the adipose and deep dermal connective tissue including destruction of vessels, nerves and epidermis. Other hallmarks of the disease are fat cell ghosts and epidermal hyperplasia. Inside the

large necrotic areas clusters of extracellular bacteria are usually focally distributed. Inflammatory responses inside the necrotic core are minimal to absent, especially in oedema and plaque lesions [40,44-46].

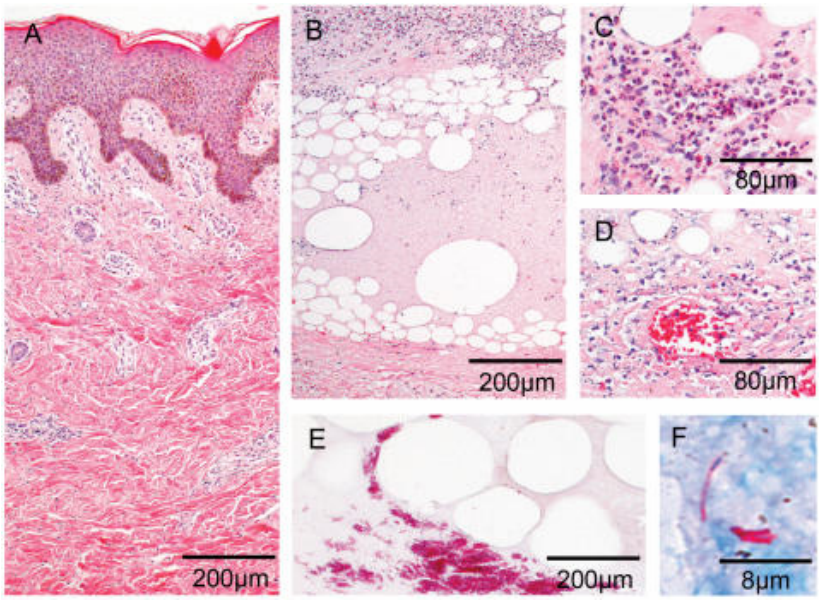

Figure 4: Histopathological characteristics associated with untreated BU lesions. (Source: Schütte et al, 2007, Plos Negl Trop Dis.)

Eventually, in late chronic stages of the disease, leukocyte infiltration and granuloma formation can be observed. The immunosuppressive effect of the toxin might be overcome by the host in late stages then allowing immunity to develop and healing to commence [47,48].

Although *M. ulcerans* is found primarily extracellular, clinical data as well as mouse studies indicate a transient intracellular stage during early infection [49,50]. Antibiotic therapy abolishes the characteristic immunosuppresion and leads to strong local inflammatory responses with the development of granulomas, ectopic lymphoid structures and the destruction of bacteria [45,51-53].

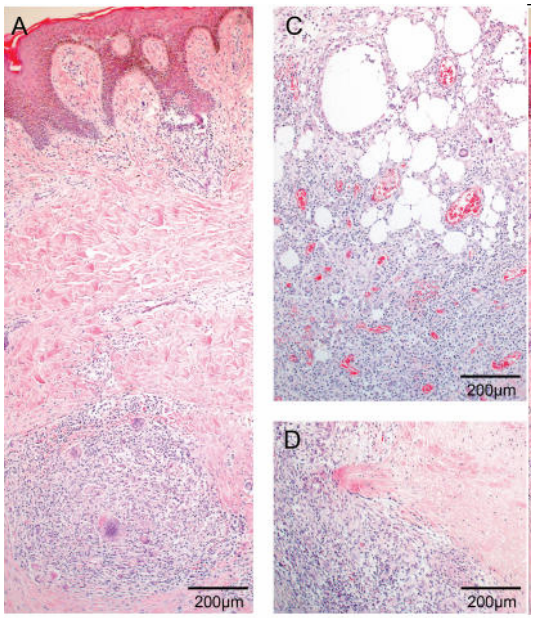

Figure 5: Histopathology of a BU lesion after rifampicin/streptomycin combination therapy. (Source: Schütte et al, 2007, Plos Negl Trop Dis.)

1.5 Immune response

Currently, only limited data on specific immune response and immune protection against *Mycobacterium ulcerans* are available. Usually, *M. ulcerans* and *M. bovis* purified protein (PPD) skin tests are negative in early stage BU patients [54,55]. Over time it may switch to a positive reaction suggesting the development of a systemic response[56,55]. New observations indicate that besides being mainly located extracellular, *M. ulceran*s proceeds through a transient intracellular phase[49,50] which may lead to the induction of a Th-1 response. Because cellular Th-1 responses with high levels of IFN-γ are regarded as crucial [57] for the hosts defence against mycobacteria several studies on T-helper subset responses were carried out, yielding controversial results. Most studies showed a predominance of Th-2 response with production of IL-10 during the early phases of the disease and a predominant Th-1 response with high secretion of IFN- γ in advanced phases as well as during healing [58-63]. In addition to the cytotoxic effects, mycolactone is also immunomodulatory in lower concentrations [64]. Demangel et al showed that mycolactone down regulates the TCR expression and alters the IL-2 production of T-cells [22].

During the extracellular stage of the disease antibodies could play a role in immunoprophylaxis and spontaneous healing. Sera from infected individuals and household contacts showed high antibody titres against different *M. ulcerans* antigens [55,65].

BCG vaccination seems to be protective in mice against low dose inoculation and in humans it seems at least to prevent the severe stage of osteomyelitis [66,67]. Repeated BCG vaccinations, attenuated *M. ulcerans* vaccine strains, and subunit vaccines incorporating protective protein antigens or the mycolactone toxin are currently under pre-clinical investigation as options for immunoprophylaxis against *M. ulcerans* disease [68].

1.6 Diagnosis

In the rural African BU endemic regions diagnosis of BU is often based alone on clinical evidence. When performed by experienced clinicians, this can be relatively reliable in particular for ulcerated lesions [54,69]. However, several other diseases can present with symptoms similar to BU. Pre-ulcerative BU lesions can be confused with lymphadenitis, mycosis, lipoma or psoriasis and ulcerative lesions with cutaneous leishmaniasis or squamous cell carcinoma [42]. Therefore, laboratory tests should be performed to confirm the diagnosis. Available tests are (1) direct smear examination by microscopy, (2) cultivation of *M. ulcerans* (3) histopathological evaluation and (4) detection of *M. ulcerans* DNA by polymerase chain reaction [40].

1.6.1 Direct smear examination

Direct smear examination is an easy, cheap and fast method to detect Ziehl-Neelsen stained AFBs under the microscope after counter-staining with methylene blue. It does not require sophisticated equipment which makes it suitable for endemic regions with poor infrastructure. However, specificity is low because several other mycobacteria positive upon staining by Ziehl-Neelsen can cause skin lesions. Therefore detection of AFB alone does not prove *M. ulcerans* to be the cause of the disease. Due to the low sensitivity (below 60%), presence of only a few bacteria might remain undetected giving a false negative result. Additionally, trained laboratory personal is needed to assure quality of the results [40,70,71].

1.6.2 Culture

Isolation of the causative agent is the final proof laboratory diagnostic method. However, cultivation of *M. ulcerans* is difficult and time- consuming. Usually, it takes between 6 weeks and 5 months to obtain a primary *M. ulcerans* culture. Besides, samples sent for cultivation are often contaminated with faster growing microorganism making a harsh decontamination necessary [13]. Sensitivity ranges between 20-60 % depending on the laboratory performing the assay [72].

1.6.3 Histopathological analysis

Samples for histopathological analysis are either small punch biopsies or surgical excisions and help to establish the differential diagnosis. There are typical histopathological features which are associated with *M. ulcerans* disease: necrosis of the subcutaneous fat tissue and formation of fat cell ghosts, oedema, epidermal hyperplasia, minimal infiltration and clusters of extracellular bacteria [44-47]. But depending on the age of a lesion or the location of the sample taken, not all hallmarks typical for BU might be present. Since bacteria are located in focal clusters in the deep subcutaneous layers, not all histopathological specimens might contain AFB. An additional challenge in the histopathological diagnosis of this disease is having adequate tissue samples, consisting of all thee skin layers (epidermis, dermis and subcutis) [40,44,70] available. When performed by well-trained personal, detection of histopathological changes is a reliable diagnostic method with a sensitivity which can be >90%.

1.6.4 Detection of *M. ulcerans DNA by PCR*

Today, PCR of *M. ulcerans* DNA, based on the amplification of the insertion sequences IS2404, IS2606 and the ketoreductase genes, is a widely used standard tool performed in national and international reference laboratories. It is a rapid, highly sensitive and specific method but requires technical expertise and is prone to contaminations. In addition to the standard PCR, real time PCR, using the TaqMan system allows to quantify *M. ulcerans* DNA providing a measure of mycobacterial burden in clinical and environmental samples [16,73-75]. Since no sufficiently sensitive point-of-care diagnostic test for BU is currently available, the WHO technical advisory

group for BU has identified development of a simple, cheap and rapid diagnostic test with high sensitivity and specificity as one of the most important research goals.

1.7 Treatment

Until 2004 the recommended treatment for BU was wide surgical excision followed by subsequent skin grafting, if necessary. Relapses occurred in 15% to 47% of the cases due to incomplete removal of the pathogen [76,77]. Studies in *M. ulcerans* infected mice revealed the combination of rifampicin and amikacin administered for 12 weeks to be active against the disease [78]. Efficacy of a combination of rifampicin and streptomycin in a clinical trial with early BU lesions led to publication of a provisional guidance by the WHO for the use of these drugs [79,80]. An observational study in Pobe, Benin in which 310 BU patients were treated, showed that nearly 50% of all lesions healed with antibiotics alone whereas others still required surgical excision and skin grafting. Altogether, the recurrence rates were as low as 1.4% [81]. These results were confirmed by several follow-up trials [82,83]. Since streptomycin has to be administered daily intramuscularly, and since there is risk of toxic side effects, especially in children, an exclusive oral treatment would be favourable. A first clinical trial conducted to compare 8 weeks of rifampicin/streptomycin with 4 weeks rifampicin/streptomycin followed by 4 weeks of rifampicin/clarithromycin showed similar efficacy [84]. In Australia BU patients treated with an all-oral chemotherapy showed healing and no recurrence [85]. More studies are needed to confirm these results and novel drugs effective against *M. ulcerans* are urgently needed because rifampicin is also one of the frontline drugs for *M. tuberculosis.*

An alternative treatment was introduced in the mid-seventies when a small trial was conducted by Meyers et al. [86]. He referred to the fact that *M. ulcerans* usually grows best between 30-32°C and therefore applied higher temperatures to the lesions. Results were encouraging but electricity requirements and complicated devices made it impossible for use in rural areas. In 2009, Junghanss et al. accomplished a first trial based on the application of heat by using a bag filled with a cheap and non-toxic phase change material which emits constant heat over hours. First results were promising, all lesions healed and no relapse was observed [87]. Currently, a large thermotherapy trial is ongoing.

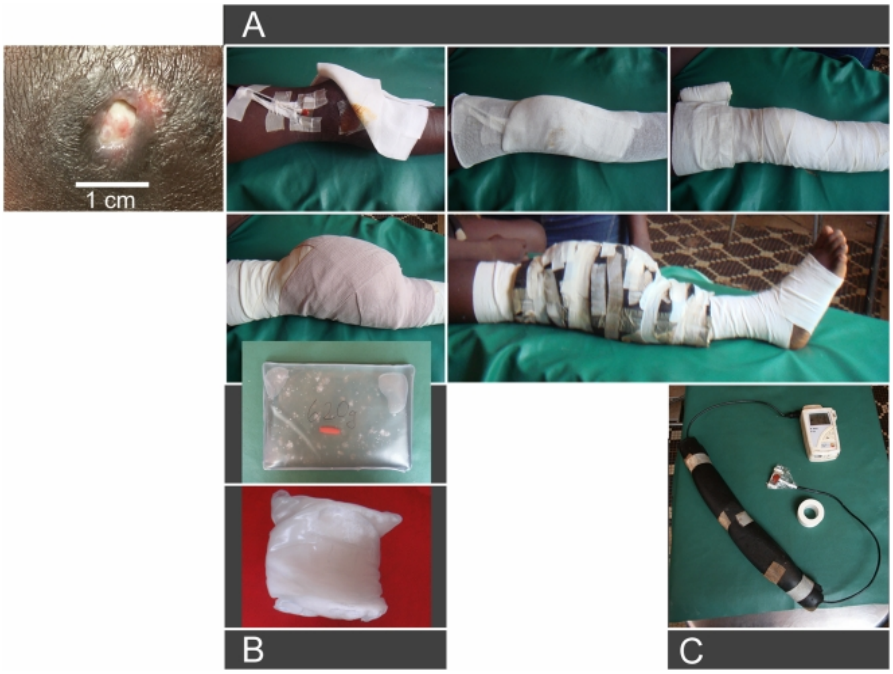

Figure 6: Mounting of the PCM-based heat application system and temperature monitoring device. (Source: Junghanss et al. 2009, Plos Negl Trop Dis.)

1.8 Wound management

Since antibiotic treatment instead of surgical excision is the primary treatment for BU, wound care and wound management becomes more and more important. Usually, patients present late with extensive ulcers and after completion of antibiotic treatment, large open wounds persist. Often skin grafting and prolonged hospital stays with intensive wound care are necessary. Delayed wound healing may be due to secondary infections or an arrest of the wound in the chronic stage. Noninfected wounds that do not progress towards closure at a consistent rate (about 10-15% wound volume per week) have to be considered as chronic wounds and need special attention and care[88] . By using limited surgical débridement to remove the dead tissue, transformation into an acute wound and initiation of the normal healing stages (inflammation, proliferation and maturation) are possible[88-91].

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Chapter 2

Goal and Objectives

2.1 Goal

The aim of this thesis was to investigate chemotherapy- associated responses, paradoxical reactions and efficacy of a new treatment regimen by histopathology, in order to gain a better insight into the host immune response and to improve treatment of *M.ulcerans* infected patients.

2.2 Objectives

1. To analyze the early local immune response and the histopathological changes during antibiotic treatment in mice experimentally infected with *Mycobacterium ulcerans*.
2. To analyze local immunological and histopathological changes during antibiotic therapy of Buruli ulcer plaque lesions and to determine the cause of ulceration during treatment.
3. To histopathologically examine and evaluate secondary lesions which occur after completion of antibiotic treatment.
4. To analyze histopathological changes in BU lesions during antibiotic therapy with two oral drugs (RIF/CLR).
5. To analyze a worldwide collection of *M. ulcerans* strains for the presence of the highly immunogenic proteins *EsxA*, *EsxB*, and H*spX*.
6. To develop and optimize a method to extract *M.ulcerans* DNA in a high yield and purity.
7. To develop a highly discriminatory fine-typing method for *M.ulcerans* and to perform microepidemiological studies in an endemic area in Ghana.

Chapter 3

Chemotherapy associated changes of histopathological features of *Mycobacterium ulcerans* lesions in a Buruli ulcer mouse model

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Abstract

Combination chemotherapy with rifampicin and streptomycin (RIF-STR) for eight weeks is currently recommended by WHO as first line treatment in *Mycobacterium ulcerans* infection (Buruli ulcer). To gain better insight into the mode of action of these antibiotics against established *M. ulcerans* infection foci and to characterize recovery of local immune responses during chemotherapy, we conducted a detailed histopathological study in *M. ulcerans* infected and RIF-STR treated mice. Mice were inoculated with *M. ulcerans* in the footpad and eleven weeks later treated with RIF-STR. Development of lesions during the first eleven weeks after infection and subsequent differences in disease progression between RIF-STR treated and untreated mice were studied. Changes in histopathological features, footpad swelling and number of colony forming units were analyzed. After inoculation with *M. ulcerans* massive infiltrates dominated by polymorphonuclear leukocytes developed at the inoculation site, but did not prevent bacterial multiplication. Huge clusters of extracellular bacteria located in large necrotic areas and surrounded by dead leukocytes developed in the untreated mice. Chemotherapy with RIF-STR led to a rapid drop in colony forming units associated with loss of solid Ziehl-Neelsen staining of acid-fast bacilli. Development of B-lymphocyte clusters and of macrophage accumulations surrounding the mycobacteria demonstrated the resolution of local immune suppression. Results demonstrate that the experimental *M. ulcerans* mouse infection model will be a valuable tool to investigate efficacy of new treatment regimens and of candidate vaccines.

Introduction

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is the third most common human mycobacterial disease of immunocompetent hosts after tuberculosis and leprosy (19, 38, 41, 42). It is a neglected emerging disease present in tropical and subtropical regions with the highest prevalence in West-African countries (2, 6). Occurrence of BU is associated with swampy areas, stagnant water bodies or slow flowing rivers. However, the mode of transmission and the natural reservoir of *M. ulcerans* are still not known. Contamination of wounds from environmental reservoirs, such as bio films on aquatic vegetation or soil, but also transmission via insect bites have been implicated (10, 24–27, 29, 30). An animal reservoir, possums, has been identified in a BU endemic region of Southern Australia (11), but so far not in Africa (9, 43).

Clinical presentation of BU starts with a painless subcutaneous nodule, papule, plaque or edema which can later develop into ulcers with extensive necrosis and undermined edges (44). The disease is primarily affecting the limbs but also other body parts can be involved. Spontaneous healing may occur often leaving extensive scarring and deformities behind (6, 38, 42). While surgery has traditionally been the only recommended treatment for BU, the current WHO treatment recommendation is a combination chemotherapy with Rifampicin and Streptomycin (RIF–STR) for 8 weeks for all forms of the active disease (3, 20, 28, 33, 45). Drawbacks of this combination are potential long term side effects and the daily intramuscular injections required for Streptomycin (37). Therefore alternative treatment regimens are being evaluated (4).

Clumps of extracellular acid fast bacilli surrounded by large areas of necrosis of the deep dermal and adipose tissue associated with only minimal inflammation are characteristic histopathological features of BU (12–14, 35). Immune responses to mycobacterial infections are normally characterized by an early, acute, predominantly neutrophilic response, whereas in the chronic stage mononuclear and granulomatous patterns develop (35). While *M. ulcerans* cells may be taken up in the early stages of infection by phagocytes, they seem to persist only transiently inside these host cells (5, 34, 36). Killing of the phagocytes by the macrolide cytotoxin mycolactone of *M. ulcerans* leads to the release of the bacteria. Subsequent extracellular multiplication

results in the development of extracellular clusters of acid-fast bacilli (AFB) inside large necrotic areas (35).

Here we have studied bacterial killing and recovery of local immune responses in a mouse model for *M. ulcerans* disease and compared these findings to features observed in human BU lesions.

Materials and Methods

Ethics statement

In conducting the experiments, the Laboratoire de Bactériologie-Hygiène followed the official instructions for the appropriate use of animals (issued by the Direction Départementale des Services Vétérinaires de Paris and the Préfecture de Police de Paris).

Infection of mice with *M. ulcerans*

The *M. ulcerans* strain CU001 isolated from a BU patient from Côte d'Ivoire was maintained through regular passage in mouse footpads. This isolate has been used in other studies evaluating the *in vivo* activity of various antimicrobial agents against *M. ulcerans*.(15–18, 23). Minimum inhibitory concentrations (MICs) of RIF and STR for strain CU001 on 7H11 agar medium are 2 and 0.25µg/ml (18). 98 female BALB/c mice, 4-week-old, were purchased from the Janvier Breeding Center, Le Genest-Saint-Isle, France. Both hind footpads of mice were inoculated subcutaneously with 0.03 ml of a freshly prepared *M. ulcerans* suspension each. The inoculum size was $8.1x10^3$ colony forming units (CFU) per footpad.

Antibiotic treatment of mice

Rifampicin (RIF; Aventis, Paris, France) was suspended in 0.05% agar-distilled water and given by oral gavage (10mg/kg body weight). Streptomycin (STR, Panpharma, Fougeres, France) was diluted with normal saline and administered by subcutaneous injection (150mg/kg body weight). Both antimicrobial agents were given once daily during weekdays, i.e., 5 times weekly. (15–18, 23)

Study design

Until week eleven after inoculation, every second week three mice were sacrificed (Figure 1). The right footpad was used for histopathological analysis whereas the left hind footpad was used to determine the CFU per footpad. After 11 weeks mice were randomized and allocated among two groups: untreated control and RIF/STR treated group. Treatment with RIF/STR was started immediately after randomization and lasted for 12 weeks. The first 4 weeks after randomization 5 mice of the treated and untreated group were sacrificed each week for histopathological analysis and

determination of CFU. Afterwards samples were taken every second week. For the untreated group two more time points (week 6 and week 8 after commencement of treatment) and for the treatment group four more time points (week 6, 8, 10 and week 12 after commencement of treatment) were evaluated (Figure 1).

Scoring of lesion index

Footpads were examined for determination of the lesion index directly before mice were sacrificed. The lesion index was scored from 0 to 4 as follows: 0, the footpad appeared normal; 1, the footpad showed slight swelling 2, swelling limited to the inoculated footpad; 3, swelling extended to the whole hind foot; 4, swelling extended to the whole limb (Figure 2).

Enumeration of colony forming units (CFU) in footpads

To enumerate CFU, the tissues of the inoculated footpads were removed aseptically and homogenized in Hanks' solution in a final volume of 2 ml. For the untreated control mice, the tissue suspensions were serially diluted in 10-fold steps, and 0.1 ml of each of three appropriate dilutions was plated in triplicate on Löwenstein-Jensen medium. For the RIF/STR treated mice, the entire volume (2 ml) of the undiluted tissue suspension from each inoculated footpad was plated on 10 tubes of Löwenstein-Jensen medium. CFU were enumerated after incubation at 30°C for 90 days.

Histopathological analyses

After mice were sacrificed, footpads were removed above the ankle and immediately fixed in neutral buffered 4 % paraformaldehyde for 24 hours. Afterwards they were incubated in decalcification solution, consisting of 0.6 M EDTA and 0.25 M citric acid, for 10 days at 37°C under shaking conditions. After decalcification of bones footpads were embedded in paraffin, cut into 5 µm sections using a HM 335 E rotary microtome (MICROM International GmbH) and retrieved on Superfrost Plus (Thermo scientific) slides. Staining with Haematoxylin/Eosin (HE) and Ziehl Neelsen (ZN) was performed according to standard WHO protocols (44).

For immunohistochemistry sections were deparaffinized and rehydrated, endogenous peroxidase was blocked with 3 % H_2O_2 for 10 minutes and unspecific binding prevented by incubating with blocking serum matching the secondary antibody host.

Subsequently slides were pre-treated with the hot Borate antigen retrieval method (0.02 M, ph 7) (21) and incubated at room temperature with (i) monoclonal antibodies against CD45R (B-cells, Serotec, Clone RA3-6B2), Mo-Ma (Monocytes/macrophages, Serotec, Clone MOMA-2), CD3 (T-cells, Serotec, Clone CD3-12), Neutrophils (Abcam, Clone NIMP-R14), KI67 (proliferation marker, Thermo Scientific, Clone SP6) or (ii) polyclonal rabbit serum against mycobacterial (*M. leprae)* antigens (pAbLep; Colorado State University, CO, USA). Afterwards sections were incubated for 30 min with a corresponding biotinylated secondary antibody (Vector Laboratories) and for additional 30 min with streptavidin horseradish peroxidase conjugate (Vectastain ABC Kit, Vector Laboratories). Staining was performed using Vector NovaRed (Vector Laboratories) and Meyer's haematoxylin as counter stain (Sigma). Sections were mounted with Eukitt® mounting medium (Fluka). Pictures were taken with a Leica® DM5000B microscope.

Based on the analysis of numerous tissue samples from lesions of Buruli ulcer patients, taken before, during or after treatment we selected characteristic pictures shown in Figure 8.

Results

Multiplication of M. ulcerans and tissue destruction in inoculated footpads

Eleven weeks after footpad inoculation with *M. ulcerans*, lesion indices of infected mouse footpads had increased from zero to “1” (Figure 2) and mice were randomized into a control group receiving no antibiotic therapy and a treatment group receiving 5 times weekly antibiotics (Figure 1). Thereafter the lesion indices of the group without antibiotic treatment proceeded fast to “3” or “4” (Figure 2). At week 19 all remaining untreated mice had to be sacrificed because first signs of severe pathology developed. These macroscopic observations correlated well with the histopathological features. Results of the histopathological analyses are shown for representative samples and for selected time points in Figure 3.

One week after inoculation, when the lesion index was still zero, only minor histopathological changes could be observed (Figure 3A1-A3). Between the muscle fibers small clusters of infiltrating cells, mainly polymorphnuclear leucocytes (PMNs) co-localizing with the AFB were observed. AFB were found both intra- and extracellularly during this early stage of infection. Animals receiving no treatment showed a swelling of the whole footpad 14 weeks after inoculation associated with large numbers of AFB, localized in accumulations of killed leucocytes (Figure 3A4-6). Infiltration in untreated footpads consisted mainly of neutrophils (Figure 4B),and monocytes/macrophages (Figure 4C), T-cells (Figure 4D) and B-cells (Figure 4E) were rarely found at this time-point of the infection the majority of bacteria were found extracellularly between the dead leucocytes. Globi-like intracellular AFB clusters (Figure 5A1, A2) may represent precursors of extracellular microcolonies (5). Without antibiotic treatment, 17 weeks after infection macroscopic swelling of footpads had extended to the ankle and the lower part of the leg (Figure 3A7-A9). Histopathology revealed large necrotic areas where connective tissue, muscles and glands were completely destroyed. Neutrophilic infiltrates present earlier during the infection had largely disappeared (Figure 3A7). Huge clusters of extracellular bacteria were primarily located in the middle of this necrotic area (Figure 3A8, A9). At the periphery of the extended necrotic areas containing this enormous bacterial burden, new foci were developing from spreading mycobacteria (Figure 5A1). Here interactions between bacterial cells and phagocytes attracted to the site of infection could be observed (Figure 5A2). In contrast, to the AFBs forming the large extracellular

conglomerations in the main necrotic infection focus (Figure 5B1, B2), more than 50% of the mycobacteria forming the new foci exhibited no longer a uniform solid ZN staining, but had a 'beaded' appearance (Figure 5A3,A4).

The epidermis above the infection foci remained intact for extended periods of time after infection, covering the necrotic dermal layer. Ulceration therefore occurred much later than footpad swelling (data not shown). Proliferation of keratinocytes in the epidermal layer was demonstrated by specific staining for the proliferation marker Ki67 (Figure 5C1, C2). Compared to tissue from uninfected mice this staining tended to be stronger, but due to heterogeneity within the dermis, a quantitative assessment of differences was not possible.

Response to chemotherapy

Footpad lesion indices of mice receiving antibiotic treatment from week eleven onwards, increased transiently to "2" in 35% of the mice or to "3" in 5% of the mice, but from week 21 onwards all footpads returned to "1" (Figure 2). While the right hind footpads were used for histopathological analysis, the left hind footpads were used for enumeration of CFUs. Chemotherapy caused a rapid decline in the number of CFUs (Figure 6). Already after three weeks of treatment four of five footpads were culture negative. However, single culture positive footpads were still found after 6 and 8 weeks of treatment and only after 10 and 12 weeks all (5/5) footpads were culture negative (Figure 6). CFU counts in untreated footpads increased further (Figure 6) until 17 weeks after inoculation when mice had to be sacrificed.

Already three weeks after start of chemotherapy, over 50% of the AFB were either internalized or associated with macrophages and neutrophils and showed no longer a uniform solid ZN staining but appeared as beaded rods (Figure 3B2, B3,). After 8 weeks of chemotherapy none of both the intracellular (Figure 7A1) as well as the extracellular AFBs (Figure 7A2) showed solid ZN staining.

Three weeks after initiation of chemotherapy, footpads showed no longer major swelling or edema, although chronic infiltrates had developed (Figure 3B1). Immunohistochemical staining revealed the formation of leukocyte accumulations mainly composed of an outer macrophage belt (Figure 7B1, B2) and an inner belt consisting of neutrophils (Figure 7B3) surrounding residual AFBs (Figure 7B4). Limited numbers of T-cells and individual B-cells were interspersed in these clusters (data not shown). While three weeks later the overall extent of the leukocyte

infiltration had decreased, very densely packed lymphocyte clusters (Figure 3B4) appeared in addition to the still persisting beaded AFBs immersed in accumulations of macrophage/neutrophils. These lymphocyte clusters (Figure 7C1) consisted mainly of CD45R positive B-cells (Figure 7C2), no macrophages (Figure 7C4) or PMNs (Figure 7C5) and only scattered T cells. More T cells were found in the areas surrounding the tightly packed clusters (Figure 7C3).

Discussion

Since 2004 WHO recommends a dual antibiotic therapy with oral rifampicin (10 mg/kg) and i. m. streptomycin (15 mg/kg) administered daily for at least eight weeks (45). While early BU lesions can usually be effectively managed by this antimicrobial treatment alone, more advanced lesions may require surgical débridement and/or skin grafting (3, 20, 28, 33). To contribute to a better understanding of the local responses in BU lesions, here, we performed a longitudinal histopathological study in mice experimentally infected with *M. ulcerans*. Changes in macroscopic appearance, mycobacterial load, tissue destruction and local immune responses before and during chemotherapy were assessed.

During the establishment of infection intracellular bacilli were observed and intracellular accumulation of AFB led to the development of globi-like structures. After killing of host cells by the macrolide toxin mycolactone these may be efficient seeds for the large extracellular clusters of AFB that developed at later stages of the infection. Evidence for a transient intracellular stage of *M. ulcerans* and the ability to subsequently destroy the infected phagocytes has also been obtained *in vitro* studies (5, 40). However, early immune responses may also have protective potential, since only a small proportion of individuals exposed to *M. ulcerans* seem to develop clinical disease (8). Immune status, as well as the size of the initial inoculums and the site of the initial infection may influence the outcome.

Animal studies have yielded conflicting results regarding the nature of leukocytic infiltrates in early stages of the infection. While it has been reported that neither wild type nor mycolactone negative *M. ulcerans* strains were strong neutrophilic attractants (1), in agreement with results reported by Coutanceau et al. (5) we observed substantial neutrophilic infiltrates during the first weeks after infection. At later stages, no viable neutrophils were left in the necrotic centre of the established BU lesions, but remains of the killed neutrophils could still be detected by immunohistochemical staining. In humans the very early stages of *M. ulcerans* infection are not associated with significant clinical signs and symptoms and have therefore never been examined histopathologically. However, massive neutrophilic infiltrates observed during the first weeks after experimental infection of mice seem to

have correlates in early human disease, since remains of neutrophils are commonly found in the necrotic areas of human BU lesions (Figure 8A-D) (32, 35).

In the mice, the epidermis above the infection foci stayed for extended periods of time after inoculation intact, covering the progressively necrotizing dermal layer. In human BU disease epidermal hyperplasia is a characteristic feature and elevated proliferation of keratinocytes is typically observed (Figure 8E, F). Also in the mice the epidermis above the infection foci showed strong staining with the proliferation marker Ki67 (Figure 5C). Other hallmarks of human BU lesions, such as extensive coagulative necrosis and edema formation (35) developed slowly in the mouse footpads and became only apparent in untreated mouse footpads about 14 weeks after inoculation. Since the mode of transmission of *M. ulcerans* is not clear, the incubation time in humans cannot be reliably estimated.

While the necrotic core of advanced BU lesions is typically free of viable leucocytes, accumulations of immune cells are found both in the mouse model and in human lesions (Figure 5A, B and Figure 8G) at the rim between the necrotic and the healthy tissue. Also in untreated lesions these seem to be sites where adaptive immune responses are initiated. These may be of importance for spontaneous healing occasionally observed both in early and advanced BU disease (7, 42).

After start of antibiotic treatment, local infiltrates switched from a predominantly unorganized neutrophilic to a more organized chronic infiltration type. Macrophages and neutrophils formed tight clusters around AFBs and dense lymphocyte clusters mainly consisting of CD45R positive B-cells developed nearby. Also in human BU lesions structured accumulations of immune cells including large CD20 positive B-cell clusters (Figure 8H) are developing in the course of chemotherapy (35). However, contrary to what is observed during antibiotic treatment of human BU lesions, we found no granuloma formation in the mouse footpads. Whether this lack of granuloma formation is related to the mouse strain used remains to be elucidated.

Already one week after start of the antibiotic treatment, incomplete ZN staining of the mycobacteria was observed giving them a beaded appearance. In *M. tuberculosis* and *M. leprae* beading seems to be an indicator of a loss of viability (22). In *M. leprae*

the morphological index (MI) is therefore used to determine the effectiveness of the antibiotic treatment. Ultrastructural visualization of *M. leprae* showed that in untreated patient samples the majority of the bacilli showed a solid staining and that cell bodies were filled homogenously with cytoplasm. In contrast irregular ZN stained bacilli from treated patients showed an intact cell wall but a cytoplasm which was detached from the cell wall and in later stages a complete degeneration of all structures (22). Beading was also detected in samples from untreated mice at the periphery of the lesions, where bacteria were found intracellularly or in close contact with phagocytes. This and similar findings in human lesions suggests that the immune system has the capacity to kill dispersed *M. ulcerans* bacteria and that development of microcolonies surrounded by a protective cloud of mycolactone is a critical step in the establishment of a chronic *M. ulcerans* infection. After completion of treatment, AFB throughout the whole footpad revealed a beaded structure, and cultures went negative supporting the hypothesis of beading as a marker for loss of viability. Osteomyelitis is a major severe complication occurring in >10% of all BU patients (31, 39). We observed in 15 % of all examined mouse footpads bacterial infiltration in the bone marrow (data not shown), but with antibiotic treatment also these bacteria revealed a beaded appearance indicating effective treatment.

Single culture positive footpads were still found after six and eight weeks of chemotherapy and only after 10 and 12 weeks all footpads were culture negative. Although also clinical trials indicate that some bacilli may survive the recommended eight week course of antibiotic treatment (28, 33), recurrence rates after RIF-STR treatment are as low as 1-2%. This indicates that immune responses primed by antigens and immunostimulators released during chemotherapy by killed bacilli are strong enough to eliminate residual dispersed bacilli (32)

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Figures

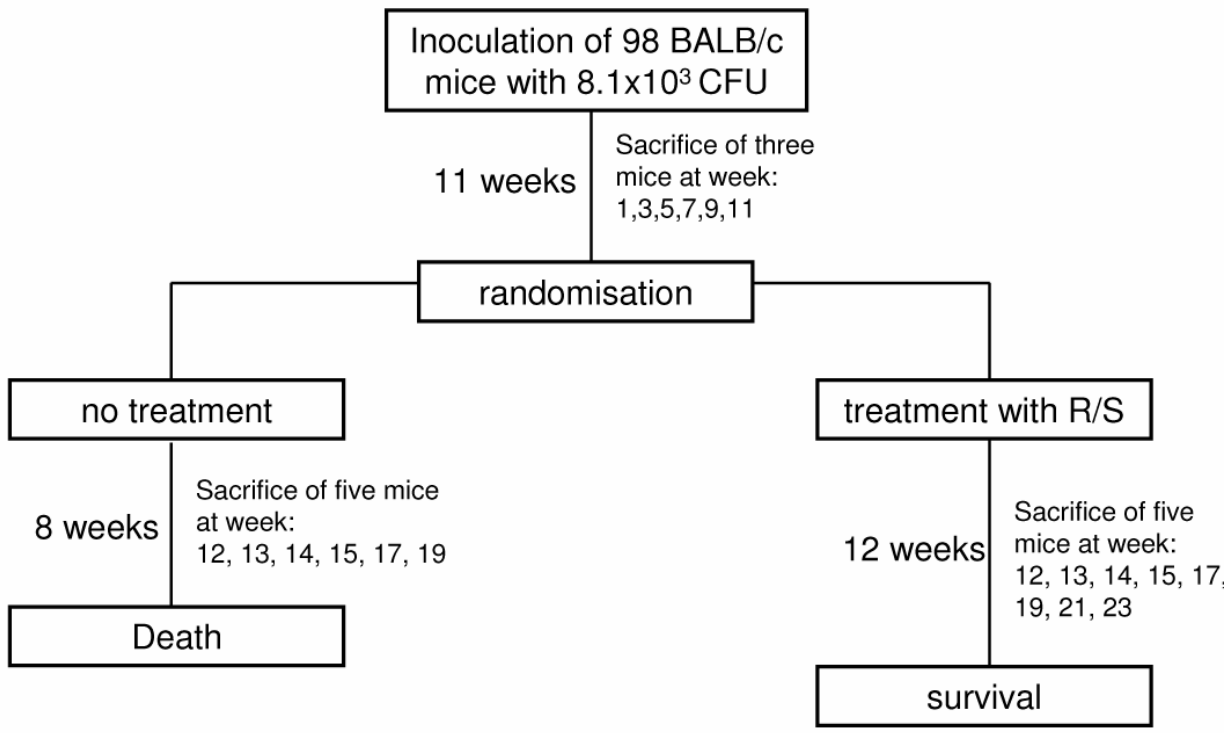

Figure 1: Study design

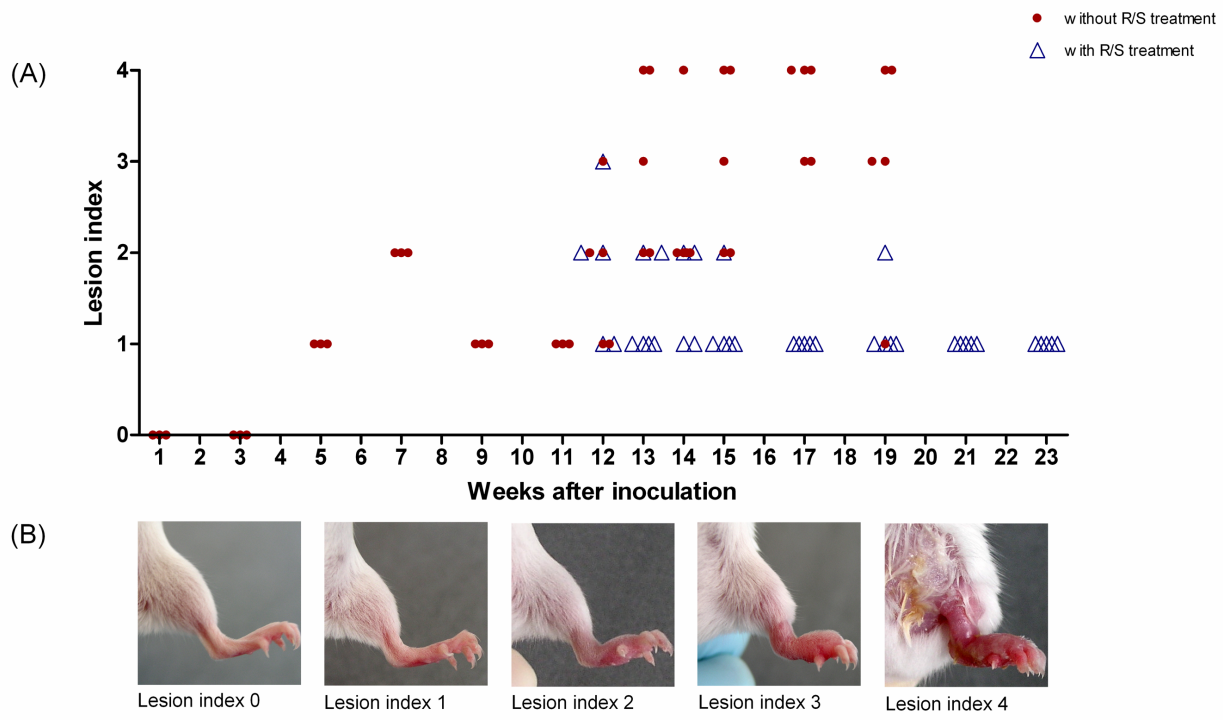

Figure 2: Evolution of the lesion index over time after infection

(A) Evolution among untreated (●) and RIF-STR treated (∆) mice. (B) Representative presentations of footpads associated with different scores of the lesion index.

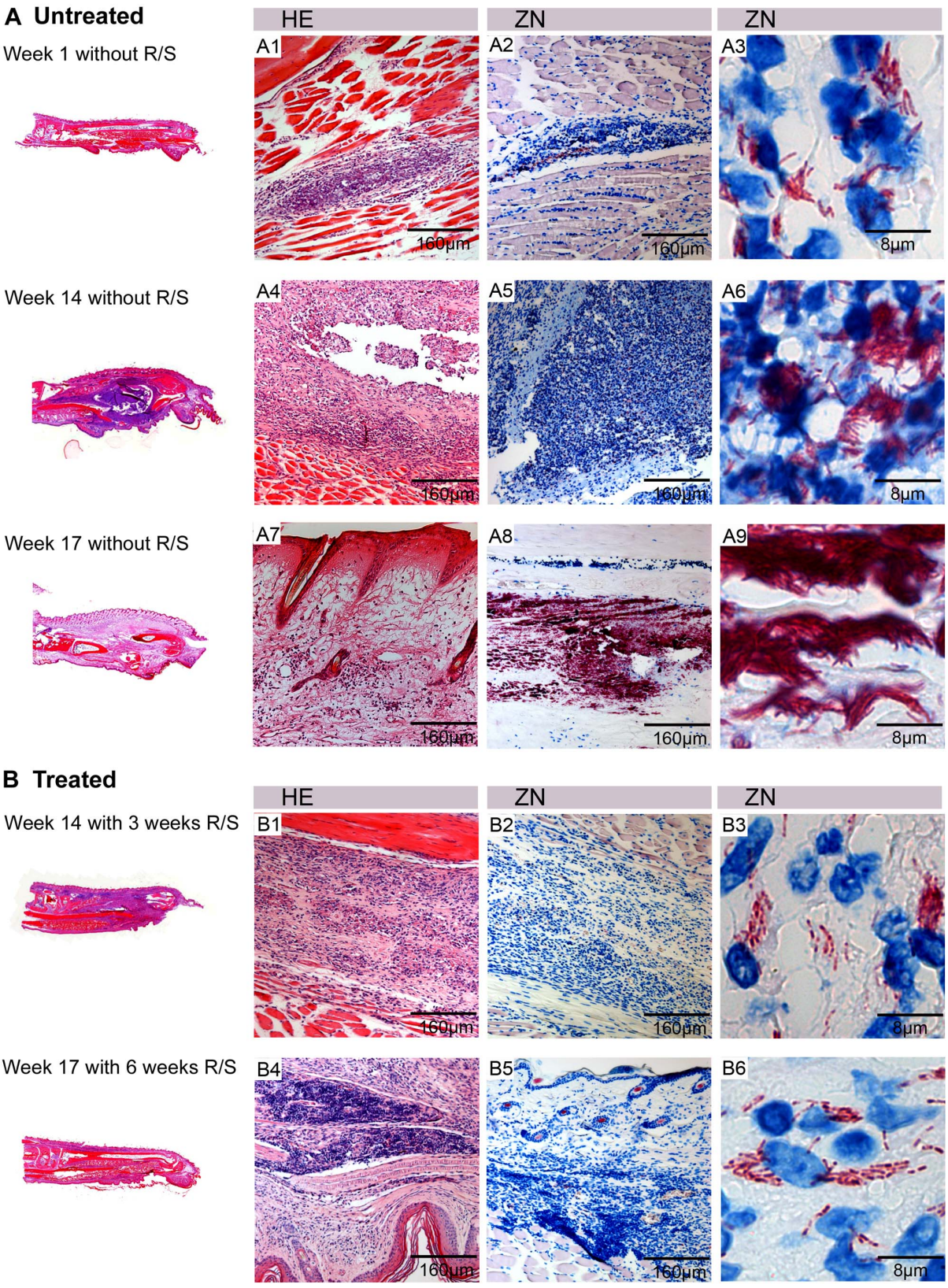

Figure 3: Overview of histopathological features of treated and untreated lesions

Histological sections stained with HE (A1, A4, A7, B1, B4) and ZN (A2, A3, A5, A6, A8, A9, B2, B3, B5, B6); counterstaining with methylenblue. Footpads from mice without chemotherapy (A) and with RIF-STR treatment (B). (A1-A3) footpad one week after inoculation, showing slight infiltration and small numbers of intra- and extracellular bacteria. (A4-A6) footpad 14 weeks after inoculation, showing a large infiltrated area mainly composed of destroyed PMNs surrounding large numbers of extracellular AFBs. (A7-A9) footpad 17 weeks after inoculation without treatment, showing a large edematous necrotic area, with extracellular bacterial clusters. (B1-3) footpad three weeks after start of treatment with RIF-STR, showing mild infiltration and AFBs with beaded appearance. (B4-B6) footpad six weeks after start of treatment, showing dense lymphocyte clusters and AFBs with beaded appearance.

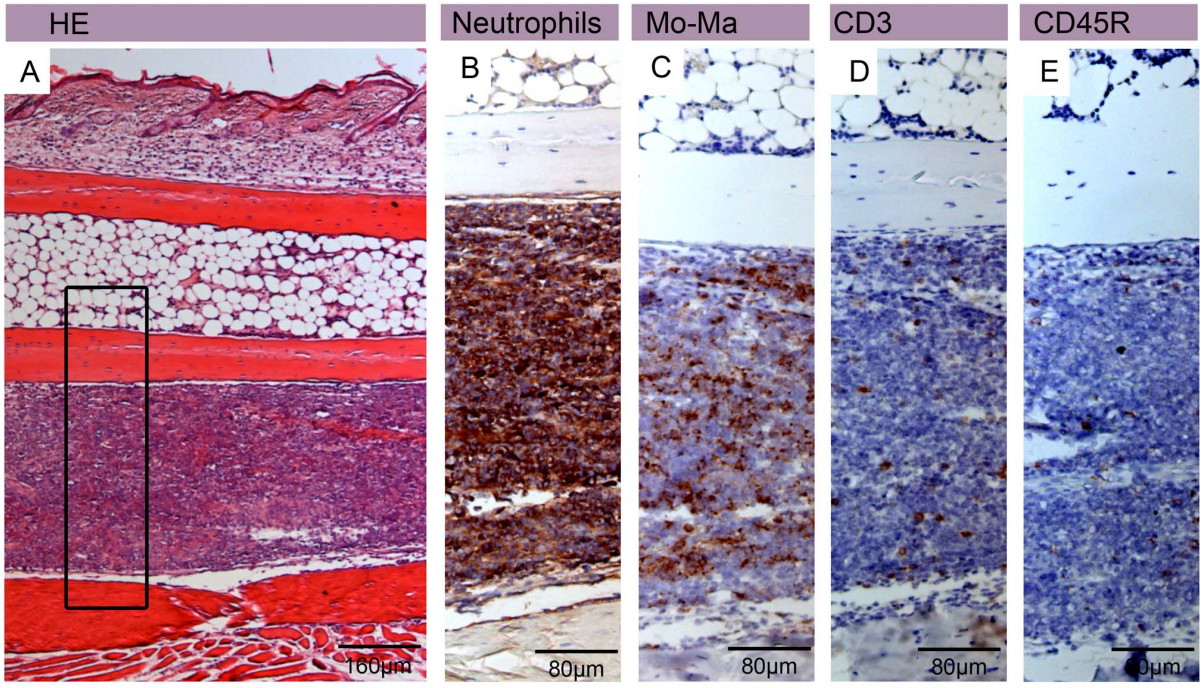

Figure 4: **Neutrophilic infiltration in untreated mouse footpads early after inoculation**

Histological sections of a footpad from an untreated mouse 3 weeks after inoculation stained either with haematoxylin-eosin (A) or with antibodies against cell surface or cytoplasmic markers and counterstained with haematoxylin (B-E). Haematoxylin-eosin staining revealed a large band of infiltrating leucocytes, mainly composed of neutrophils (B) and monocytes/macrophages (C). Only few CD3 positive T- cells (D) and few CD45R positive B-cells (E) were found.

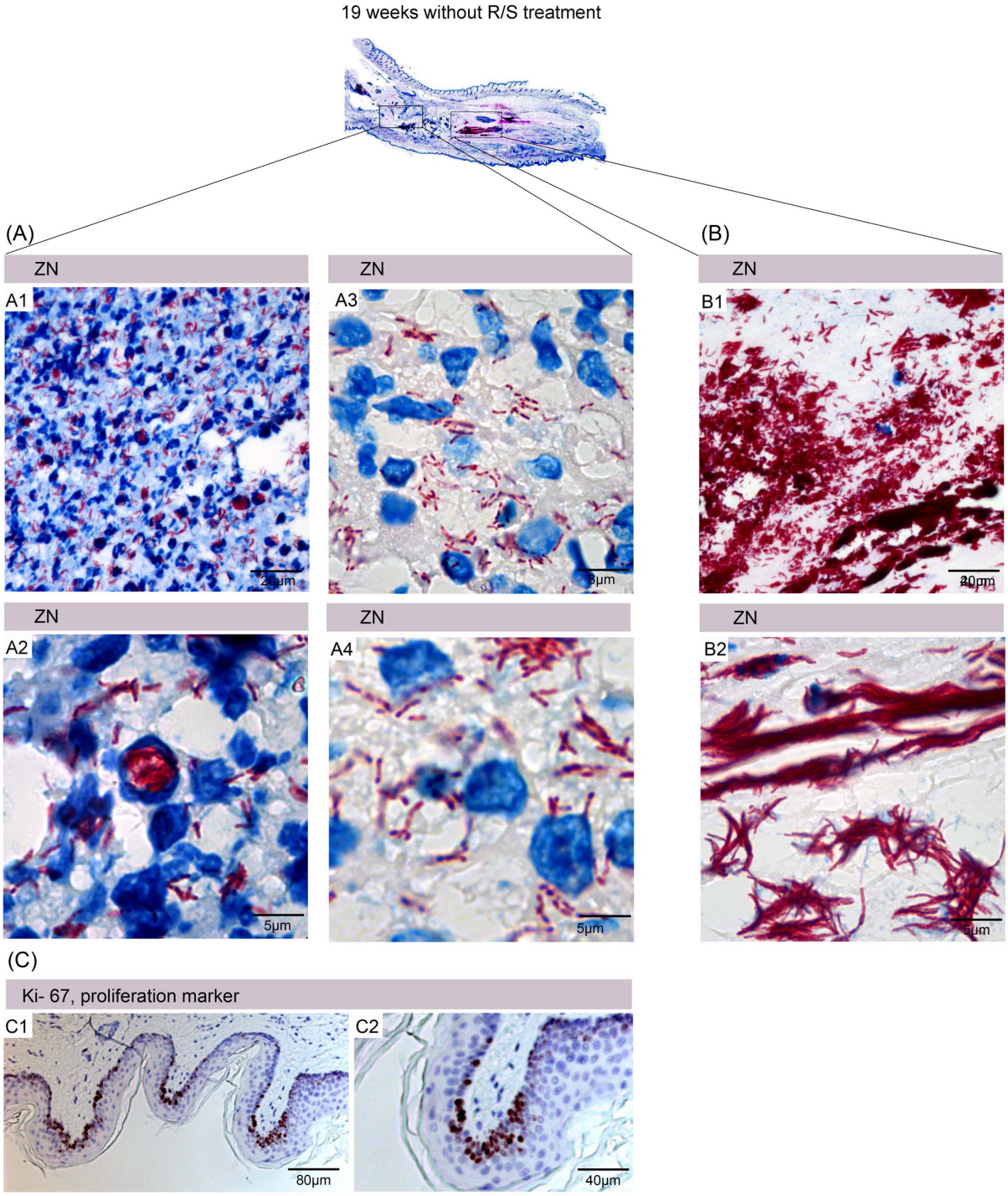

Figure 5: Appearance of mycobacteria at different times after inoculation Histological sections of the footpad from an untreated mouse 19 weeks after inoculation (stained with ZN, counterstained with methylene blue (A, B) or with an antibody-based staining, counterstained with haematoxylin (C). The main infection focus and a more peripheral accumulation of AFBs are boxed (A). Low magnification picture of a peripheral AFB accumulation, showing interactions between the

mycobacteria and phagocytes (A1). Globi-like structures representing numerous intracellular AFB residing in macrophages (A2). ZN staining of peripheral AFB reveals the presence of beaded bacteria (A3, A4). The main infection focus containing no viable phagocytes, but large extracellular clusters of AFB (B1) exhibiting solid ZN staining (B2). Staining with the proliferation marker Ki67 revealed a strong proliferative activity of epidermal kerationocytes (C1, C2).

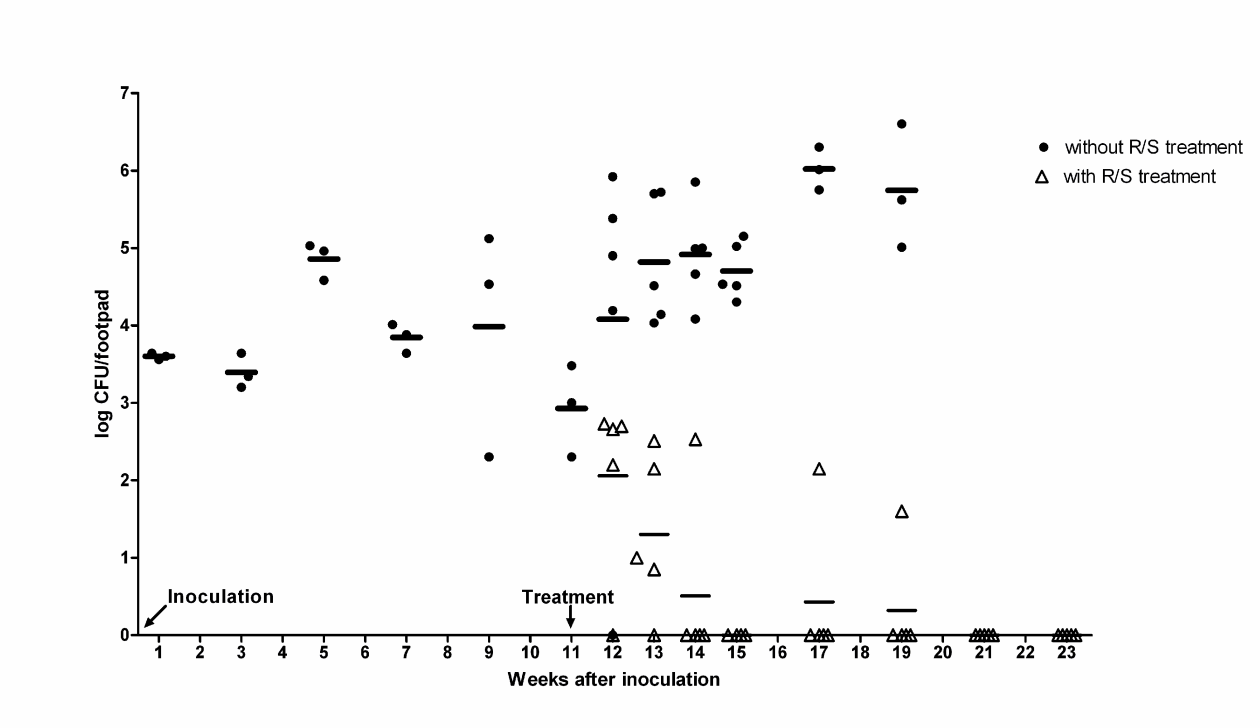

Figure 6: Changes in CFU counts after inoculation and during RIF-STR treatment

Mice footpads were inoculated with $8.1x10^3$ CFU *M. ulcerans* at week 0. The control group (●) received no treatment; in the treatment group (Δ), chemotherapy with RIF-STR started at week 11.

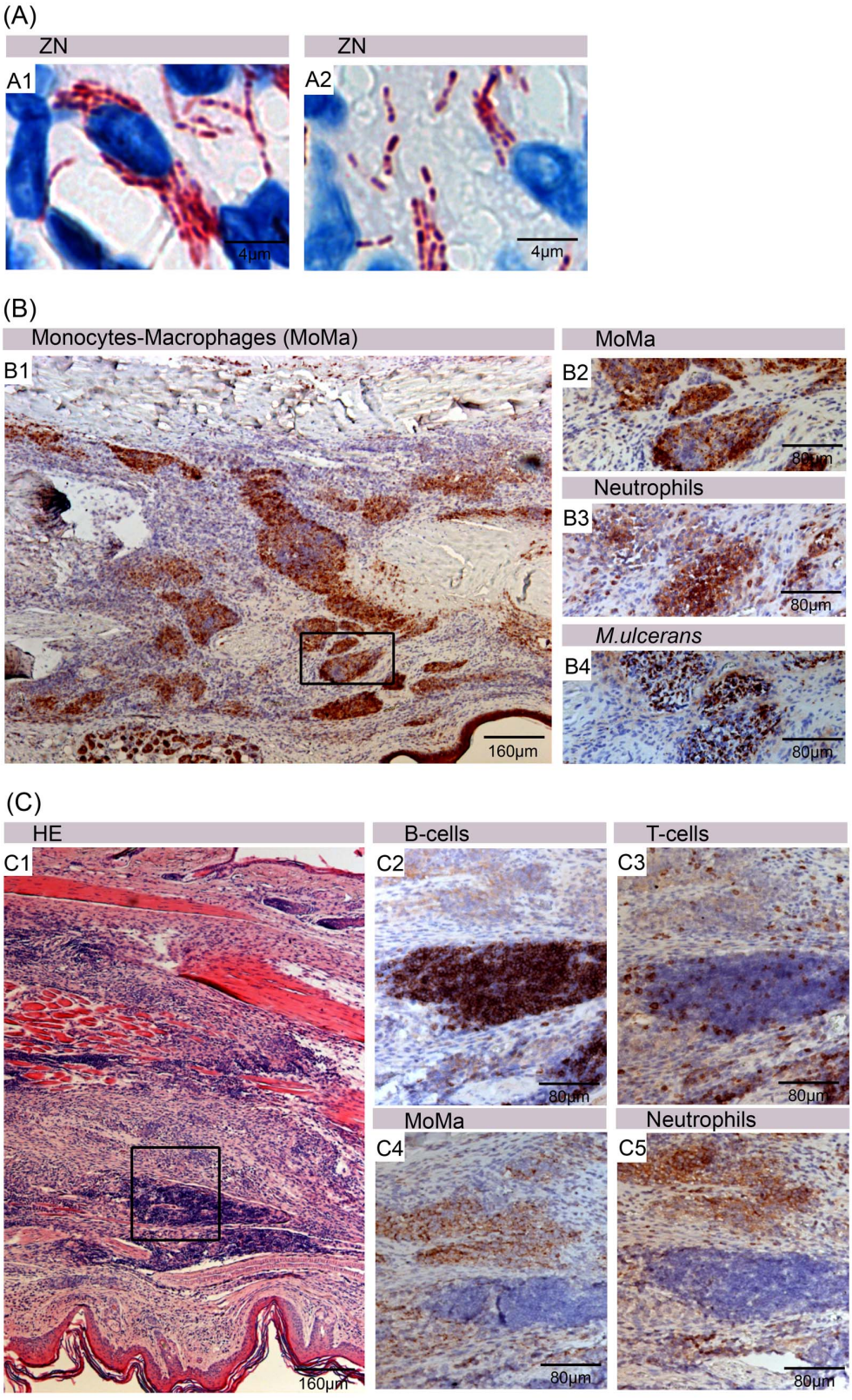

Figure 7: Inflammatory responses and development of B-cell clusters in RIF-STR treated mice

Histological sections were either stained with ZN-methylenblue (A1,A2), antibodies against cell surface or cytoplasmic markers, counterstained with haematoxylin (B1-B4, C2-C5) or sections were stained with Haematoxylin/Eosin (C1). After 8 weeks of antibiotic therapy all intracellular (A1) as well as extracellular (A2) AFB appeared as beaded rods. Immunostaining of mouse footpads three weeks after commencement of antibiotic therapy revealed a strong clustering of monocytes/macrophages (Mo-Ma) (B1, B2), neutrophils (B3) and *M. ulcerans* bacteria (B4), as well as the formation of large lymphocyte accumulations six weeks after commencement of antibiotic therapy (C) which were mainly composed of CD45 positive B-cells (C2), only a few CD3 positive T-cells C3) and basically no macrophages (C4) or neutrophils (C5).

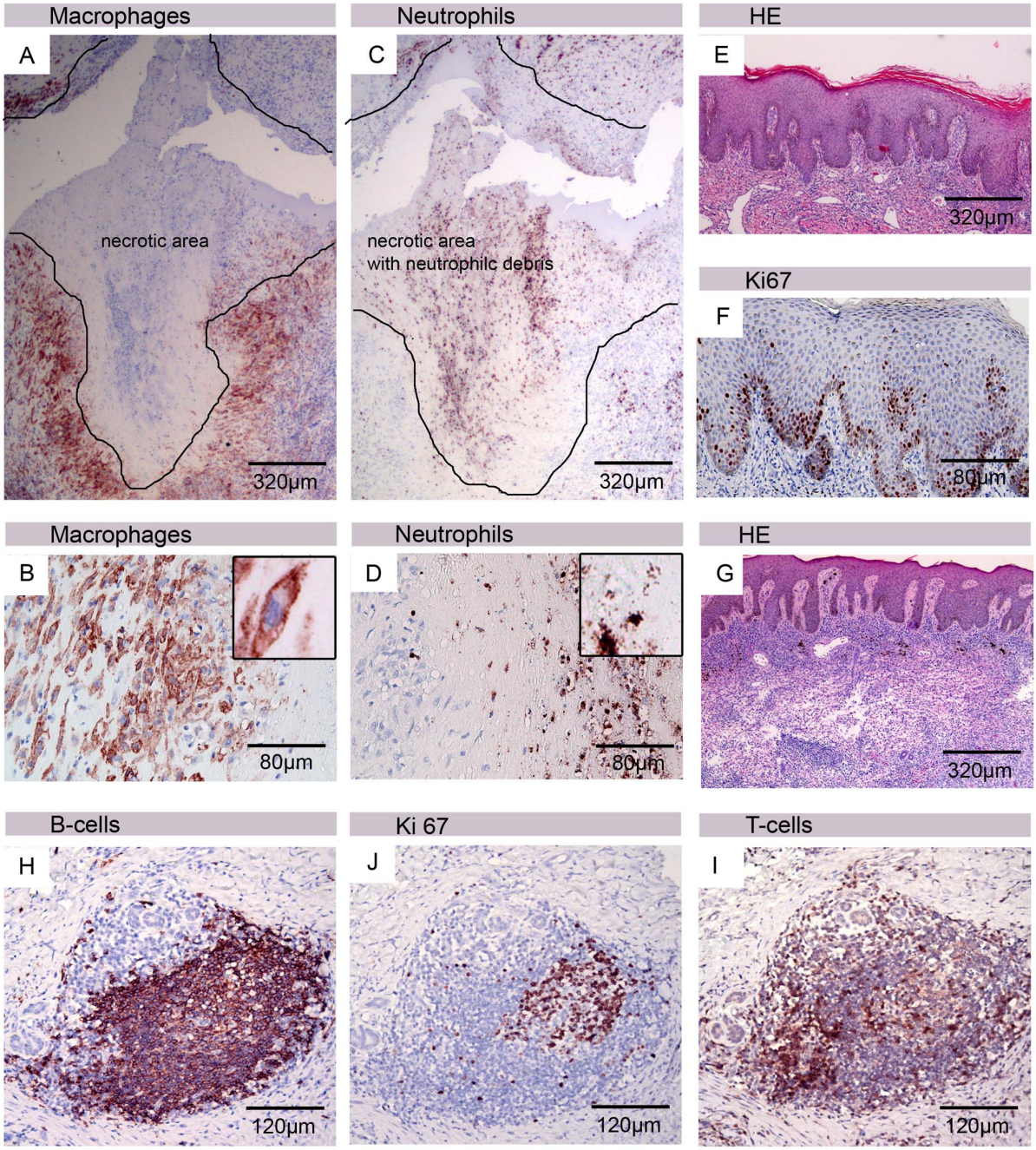

Figure 8: Features of human BU lesion with correlates in the mouse infection model

Histological sections of human tissue were either stained with haematoxylin-eosin (E,G) or with antibodies against cell surface or cytoplasmic markers and counterstained with haematoxylin (A-D, F, H-J). A belt of intact macrophages surrounded the necrotic core (A) with neutrophilic debris from the initial wave of infiltration still being detectable (C). Appendices of macrophages reaching into the necrotic core (B). Inside the necrotic area no intact neutrophils could be detected (D).

Epidermal hyperplasia typically observed in human BU lesions (E) associated with an elevated proliferation of the keratinocytes, indicated by staining with the Ki67 proliferation marker (F). In the neighborhood of the necrotic core of the lesion, accumulations of viable leucocytes can be observed in untreated patients (G). Cluster of CD20 positive B-cells (H), partially highly proliferating (I) and interspersed with few CD3 positive T-cells (J) are typically developing in the lesions of BU patients during and after completion of chemotherapy.

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Chapter 4

Histopathological changes and clinical responses of Buruli Ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue?

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Abstract

Background: Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a necrotizing skin disease usually starting with a subcutaneous nodule or plaque which may ulcerate and progress, if untreated, over months and years. During the currently recommended antibiotic treatment with rifampicin/streptomycin plaque lesions tend to ulcerate, often associated with retarded wound healing and prolonged hospital stays.

Methodology/Principal findings: Included in this study were twelve laboratory reconfirmed, HIV negative BU patients presenting with plaque lesions at the CDTUB in Allada, Benin. Punch biopsies for histopathological and immunohistochemical analysis were taken before start of treatment and after four to five weeks of treatment. Where excision or wound débridement was clinically indicated, the removed tissue was also analyzed. Based on clinical judgment, nine of the twelve patients enrolled in this study received limited surgical excision seven to 39 days after completion of chemotherapy, followed by skin grafting. Lesions of three patients healed without further intervention. Before treatment, plaque lesions were characterized by a destroyed subcutis with extensive necrosis without major signs of infiltration. After completion of antibiotic treatment partial infiltration of the affected tissue was observed, but large necrotic areas remained unchanged.

Conclusion/Significance: Our histopathological analyses show that ulceration of plaque lesions during antibiotic treatment do not represent a failure to respond on antimycobacterial treatment. Based on our results we suggest formal testing in a controlled clinical trial setting, whether limited surgical excision of necrotic tissue favours wound healing and can reduce the duration of hospital stays.

Author Summary

The tropical necrotizing skin disease Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is associated with extensive tissue destruction and local immunosuppression caused by the macrolide exotoxin mycolactone. Chemotherapy with a combination of rifampicin and streptomycin for 8 weeks is the currently recommended treatment for all types of BU lesions, including both ulcerative and non-ulcerative stages (plaques, nodules and oedema). Our histopathological analysis of twelve BU plaque lesions revealed extensive destruction of sub-cutaneous tissue. This frequently led to ulceration during antibiotic treatment. This should not be mistaken as a failure of the antimycobacterial chemotherapy, since we found no evidence for the persistence of active infection foci. Large necrotic areas were found to persist even after completion of antibiotic treatment. These may disturb wound healing and the role of wound débridement should therefore be formally tested in a clinical trial setting.

Introduction

Buruli ulcer (BU), the third most common human mycobacterial disease, is caused by *M. ulcerans* [1,2]. While the disease is present in around 30 countries worldwide the main focus with the highest prevalence is found in West African countries like Benin, Ghana, Cameroon and the Ivory Coast [3,4]. Three categories of pre-ulcerative lesions, painless movable subcutaneous nodules or papules, oedema and plaques are distinguished. All three forms of pre-ulcerative lesions may progress to ulcerative lesions, when destruction of the subcutis is leading to the collapse of the overlying epidermis and dermis [1,5,6].

In 2004 WHO treatment recommendations for BU changed from a purely surgical treatment to a dual antibiotic therapy with rifampicin and streptomycin for eight weeks [7]. Recurrence rates after antibiotic treatment are low, but a proportion of antibiotic treated patients, in particular those with extensively ulcerated wounds, requires excisions and skin grafting [8-13]. During and after completion of antibiotic treatment paradoxical reactions associated with the enhancement of local immune responses and increases in size of lesions may be mistaken as disease progression [14,15]. Observational studies have shown that while nodules usually heal after antibiotic treatment without further intervention, ulceration and an increase in the size of the lesion is often observed in the case of plaque lesions. These paradoxical reactions may occur after initial improvement and often require extensive medical care, causing long hospital stays. To elucidate the underlying mechanisms and to gain a better insight into the histopathological features of plaque lesions we conducted detailed histopathological and immunohistochemical analyses of tissue specimen from 12 plaque patients treated with rifampicin and streptomycin.

Materials and Methods

Ethics statement

Ethical approval (clearance N° 011, 12/10/2010) for analyzing patient specimens was obtained from the provisional national ethical review board of the Ministry of Health Benin, registered under the N° IRB00006860. Written informed consent from the patients or from the guardians of the patients was obtained before surgical specimens were used for reconfirmation of clinical diagnosis and detailed immunohistological analysis.

Study participants

12 patients aged between five and 70 years (median age 12 years) reporting between April 16 and August 15 2009 with laboratory reconfirmed BU plaque lesions at the Centre de Depistage et de Traitement de l'Ulcere de Buruli d'Allada in Benin were included in the study (Table 1). Most (9/12) lesions were located at the upper (4/12) or lower (5/12) extremities. The diameter of the lesions was between four and 15 cm. All patients were coming from the highly BU endemic Ze commune in the Department Atlantique of Benin. The gender distribution was nine males and three females. Clinical diagnosis was reconfirmed by positive results in at least two of the three laboratory tests (*IS2404* PCR, detection of acid fast bacilli (AFBs) on microscopy and histopathology) performed. All patients completed the WHO recommended combination dual chemotherapy with oral rifampicin (10 mg/kg/day) and i. m. streptomycin (15 mg/kg/day) for 56 days. All patients were tested negative for HIV.

Histopathological analysis

Punch biopsies were taken for histopathological analyses prior to start of chemotherapy on day -2 to day 0 (T1). Of these 12 T1 samples, one was not suitable for immunohistochemical analysis. A second punch biopsy taken 26 to 48 days after start of chemotherapy (T2) became available from 11/12 patients. According to the judgment of the responsible clinician, based on the evolution of the lesions including remaining induration and increasing lesion surface area, nine patients received adjunct surgical treatment seven to 39 days after completion of chemotherapy and had skin grafting five to 14 days after excision. Samples from seven of the nine

excised lesions became available for histopathological analysis. In the case of the two other patients that received surgery, a third punch biopsy was taken and analyzed prior to surgical excision. Tissue samples were fixed in 4% neutral-buffered paraformaldehyde for 24h and subsequently transferred to 70% ethanol for transport. Biopsies were dehydrated, embedded in paraffin, cut into 5 mm thin sections and retrieved on glass slides. After dewaxing and rehydration, sections were stained with haematoxylin/eosin (HE) and Ziehl-Neelsen (ZN) staining of AFBs was performed. Immunohistochemistry was performed with antibodies against Elastase (polymorphonuclear neutrophils [PMNs]; Dako clone NP57), CD3 (T lymphocytes; Dako clone F7.2.38), CD8 (cytotoxic T lymphocytes; Serotec clone 4B11), CD4 (helper T lymphocytes; Dako clone 4B12), CD68 (macrophages/monocytes; Dako clone KP1), Ki67 (proliferation marker; Dako polyclonal rabbit serum) and CD20 (B lymphocytes; Novocastra clone7D1). Staining was performed using Vector NovaRED and haematoxylin as a counterstain.

Results

Clinical response to antibiotic treatment

Included in this study were twelve BU patients (Tab. 1) with single new laboratory-reconfirmed plaque lesions reporting between April 16 and August 15, 2009 at the Centre de Depistage et de Traitement de l'Ulcere de Buruli d'Allada in Benin. All patients received the WHO recommended dual chemotherapy with rifampicin and streptomycin for 56 days [7]. Seven out of 12 lesions ulcerated during chemotherapy (Fig. 1C, E, F). Six of these patients received surgical treatment to remove necrotic tissue (Fig. 1E, F). Based on clinical judgment, tissue was also excised from the lesions of another three patients, which had at this stage not yet developed ulceration, but an induration (Fig 1D). Three patients healed without surgical intervention (Fig. 1A-C), one of them had developed a small ulceration during chemotherapy (Fig. 1C) which needed no further intervention.

For patients who received surgical treatment, excisions were performed 7 to 39 days (average 19 days) after completion of chemotherapy. Skin grafting followed 5 to 14 days (average 8 days) after excision and patients were discharged from hospital 16 to 100 days (average 36 days) after skin grafting. The time interval between start of antibiotic treatment till discharge from hospital was between 55 to 179 days (average 103 days) for the 12 patients (Tab.1). Depending on the location of the lesion this period was prolonged by a phase of physiotherapy (Tab. 1).

Histopathological features of untreated plaque lesions

For histopathological characterization of the untreated plaque lesions, punch biopsies were taken before start of antibiotic treatment. Certain features, depicted in Fig. 2, were found in all samples analyzed. The dermis presented with relatively intact collagen with minor infiltrations around vessels and glands (Fig. 2A, C), reflecting the pre-ulcerative nature of the plaque lesions. Most strikingly, the subcutis was in all patients extensively necrotic and oedematous (Fig. 2A). Additional features typical for an untreated BU lesion, like fat cell ghosts (Fig. 2D) and minimal infiltration limited to the surrounding of a few remaining partially intact blood vessels (Fig. 2E), were always present. Immunohistochemical staining revealed N-elastase positive neutrophilic debris (Fig. 2F) reflecting an early wave of neutrophilic infiltration. In addition, only few intact neutrophils (Fig. 2G) and CD68 positive macrophages (Fig.

2H) were found. Tissue of only two patients contained also a few intact CD3 positive T-cells in the dermal tissue (data not shown).

Acid fast bacilli (AFB) were found in only 7/31 (23%) of the tissue samples analyzed altogether. This reflects the focal distribution of the mycobacteria and the extension of tissue destruction, attributable to the diffusion of mycolactone, into tissue areas with low mycobacterial burden [16]. Fig. 2B depicts an example, where a mycobacterial focus was sampled. Here a band of extracellular AFBs was found in a deep layer of the necrotic subcutis.

Persistence of necrotic areas with limited infiltration in the course of antibiotic treatment

Punch biopsies taken between 26 and 48 days after start of antibiotic treatment typically consisted still primarily of large oedematous necrotic areas with fat cell ghosts (Fig. 3A/B). Overall, infiltration was much less pronounced than typically found in ulcerative lesions at this time point of antibiotic treatment [17]. 9/11 patients presented with a mild infiltration consisting of very few N-elastase positive neutrophils (Fig. 3D), more CD68 positive macrophages (Fig. 3E) and CD3 positive T-cells (Fig. 3F). These infiltrates were scattered throughout the dermis and extended only in 3/11 samples into the large necrotic areas. Structured infiltrates typically found in healing BU lesions [14], were rare: granuloma formation (Fig. 3H) was found in 2/11 samples, CD20 positive B-cell clusters (Fig. 3G) and giant cells (Fig. 3I) in 3/11 biopsies. AFBs were found in 4/11 samples; they were primarily intracellular or had a ‘beaded’ appearance (Fig. 3C).

Features of tissue excised after completion of antibiotic treatment

While lesions of three of the enrolled patients healed without adjunct treatment, the responsible clinician decided to support wound healing by surgical excision of affected tissue in 9/12 patients. All nine excisions were performed after completion of antibiotic treatment, 56 to 94 days after start of chemotherapy. While six of the nine tissue samples were excised from lesions, which had spontaneously ulcerated during antibiotic treatment, the other three were excised from non-ulcerated lesions showing no adequate clinical improvement.

All nine excisions consisted to a large extent of necrotic and oedematous tissue with fat cell ghosts (Fig. 4A). In the case of the three patients which had still non-

ulcerative lesions at the time of excision, the dermis presented with necrosis and infiltration, indicative for progression towards ulceration (Fig. 4C). Most (7/9) samples showed massive infiltration of the subcutis (Fig. 4B) often with a clear border between intact leucocytes, mainly CD14 positive macrophages/monocytes (Fig. 4D1), and the still necrotic areas containing N-elastase positive neutrophilic debris (Fig. 4D2). Infiltrates were mainly composed of CD68 positive macrophages (Fig. 4E) and CD3 positive T cells (Fig. 4F) with a higher proportion of CD8 positive (Fig. 4G) than CD4 positive (Fig. 4H) T-cells. In addition CD68 positive langhans and foreign body giant cells (Fig. 4I), granulomas and small CD20 positive B-cell clusters (Fig. 4J) were found. Some areas were strongly infiltrated with N-elastase positive neutrophils (Fig. 4K). Angiogenesis inside the necrotic and hypoxic tissue was observed in 5/9 patients (data not shown). Small numbers of intra- and extracellular AFB with a beaded appearance were found in the specimens of 2/9 patients (Fig. 4L).

Discussion

BU plaque lesions are defined as firm, painless, elevated and well demarcated lesion with more than 2 cm in diameter [18]. Our histopathological analysis of the tissue specimen from plaque lesions reconfirmed earlier quantitative RT-PCR analyses [16], demonstrating a focal distribution of the mycobacteria and a mycolactone-mediated extension of tissue destruction to tissue areas with low mycobacterial burden. Our studies provided no evidence for survival of mycobacterial clusters after chemotherapy. The few AFBs found after chemotherapy had beaded appearance and were largely phagocytosed [17] and all tissues turned culture negative during treatment. Interestingly, in those punch biopsies of untreated BU lesions, where a mycobacterial focus was sampled, mycobacteria were typically found as a band of extracellular AFBs in a deep layer - several mm below the epidermis- of the necrotic subcutis (Fig 2B). This might explain why in cases, with clinical features consistent with BU, microscopic results are frequently and PCR results are occasionally negative. Especially when collecting diagnostic specimens from non-ulcerative lesions by fine needle aspiration, also deep layers should be sampled to increase chances to reach these bacteria. Focal distribution of *M. ulcerans* and related lack of AFB in some of the tissue samples reflects a major limitation of histopathological analyses using punch biopsies. While findings at a particular location of the lesion may not be representative for the entire lesion, many major histopathological features described here were very consistently found in all samples analyzed.

Also earlier studies [14] demonstrating a reversal of local immune suppression during chemotherapy were confirmed. This process starts with a diffuse chronic infiltration, primarily by macrophages and T cells. While neutrophils play only a minor role in this process, N-elastase positive debris in the necrotic areas are indicative for a wave of neutrophil invasion during the early phase of BU pathogenesis. It has been shown [14] that after an initial phase of diffuse infiltration development of structured leukocyte aggregates, such as B cell clusters and granulomas usually are observed. While the development of such highly organized ectopic lymphoid tissue was also observed in the case of plaque lesions, this was confined to the margins of the necrotic areas. Large regions showing massive coagulative necrosis without

significant infiltration were still found in the surgical specimens excised 7 – 39 days after completion of chemotherapy.

Infiltration and angiogenesis in the affected tissue during and after chemotherapy promotes the resorption of tissue debris. Initial inflammatory responses may be associated with paradoxical reactions, before converging into a phase of wound healing. In two of the 12 patients enrolled, this process of resorption of necrotic tissue was efficient enough to permit healing without ulceration. However, in most cases the necrotic areas of plaque lesions seemed to be too extensive to permit complete resorption without ulceration. In general, our histopathological analysis of plaque lesions revealed a much larger and deeper destruction of the subcutaneous tissue than expected. Spontaneous ulceration during antibiotic treatment was observed in 7/12 patients and tissue samples from three other non-ulcerated patients showed gradual degeneration of the dermis, indicative for a progression to ulceration. While ulceration result in the loss of necrotic tissue, our analysis of tissues surgically excised 7 – 39 days after completion of chemotherapy from lesions that spontaneously ulcerated during chemotherapy, revealed incomplete loss of necrotic tissue. These findings support the decision of the responsible clinician to support wound healing by débridement of the margins of the ulcers. It is well documented that wound débridement i.e. the removal of materials incompatible with healing, can substantially accelerate the complex wound healing process [19-21]. Even if superinfections are controlled with antibiotics, chronic wounds can be caught in a chronic inflammatory phase and débridement is then required to convert the chronic wound bed into an acute wound and mediate healing through the stages of inflammation, proliferation and maturation [20]. This may also apply for BU lesions which show massive infiltration during antibiotic treatment and may subsequently be arrested in a chronic stage without the chance of proper healing. In patients with severe and extensive lesions an early decision for wound débridement may therefore reduce hospital stays to less than 100 days.

In the present study the time span between start of treatment and discharge from the hospital was 55 - 61 days for the three patients, which did not require an excision. For those three patients, which showed no spontaneous ulceration, but were surgically treated to remove necrotic tissue this time span was 90 – 95 days.

Degeneration of the dermis of the excised lesions harbouring large areas of necrotic tissue indicated that these patients would have developed ulceration at a later stage, leading to a severely delayed subsequent start of the healing process. Those six patients who showed spontaneous ulceration during chemotherapy and received later wound débridement, stayed for 108 – 179 days in the hospital. Surgical treatment was performed 7 – 39 days after completion of chemotherapy and skin grafting 5 – 7 days after excision. Five of these six patients were discharged from hospital 27 – 38 days after skin grafting, only in one case this time period was much longer (99d) due to secondary infection and delayed wound healing. While bacteria are present on basically every open wound, secondary infections above a critical bacterial load (>10^5 organisms per gram of tissue) may lead to an arrest of the wound healing process [22-24].

Taken together our analysis indicates that due to massive coagulative necrosis only a minority of BU plaque lesions may heal without spontaneous ulceration. It appears advisable to consider débridement of ulcers that have developed in the course of chemotherapy in order to remove necrotic tissue and to favour wound healing. The effect of adjunct surgical treatment should therefore be formally tested in a clinical trial setting to support development of differentiated guidelines for BU wound management.

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Table

Table 1: Characteristics of the 12 Buruli ulcer plaque patients

Table 1. Characteristics of the 12 Buruli ulcer plaque patients.

Patient	Ulceration during chemotherapy*	Time span between end of chemotherapy and excision	Time span between excision and skin grafting	Time span between skin grafting and discharge	Time span between start of treatment and discharge	Sex	Age (years)	Site of lesion	Lesion size at admission
1	no	no	no	no	61	M	5	thorax	6 cm×4 cm
2	no	no	no	no	55	M	12	trunk	10 cm×7 cm
3	yes	no	no	no	55	M	5	face	7 cm×7 cm
4	no	10	14	16	95	F	13	upper leg	9 cm×8 cm
5	no	10	7	18	90	M	20	foot	5 cm ×4 cm
6	no	10	7	23	95	M	15	elbow	5 cm×5 cm
7	yes	10	7	36	108	F	70	knee	8 cm×5 cm
8	yes	7	7	33	108	M	32	upper leg	15 cm×14 cm
9	yes	17	7	100	179	M	5	lower arm	12 cm×10 cm
10	yes	39	5	31	130	M	12	hand	12 cm×13 cm
11	yes	31	7	36	129	M	12	lower leg	10 cm×8 cm
12	yes	39	7	28	129	F	9	elbow	7 cm×5 cm

*the mean duration from start of treatment to ulceration was 30 days (11–53 days) for those five patients for which beginning of ulceration could be exactly recorded.
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Figures

Figure 1: Presentation of BU lesions before and during therapy

Representatives of the different patient groups are depicted. A, B: Patients with lesions that did not ulcerate and which did not require a surgical intervention. C: Patient with a lesion that ulcerated during treatment but did not require a surgical intervention. D: Patient with a lesion that did not ulcerate but was excised; E, F: Patients with lesions that ulcerated during treatment and needed surgical excisions.

A: Patient 1; day -1, before start of treatment, punch hole visible. B: Patient 2; day 0, at the start of treatment, punch hole visible. C: Patient 3; C1: day -2 before start of treatment; C2: day 50 after start of treatment; C3: day 300 after end of treatment. D: Patient 5; D1: day -8 before start of treatment; D2: day 10 after end of antibiotic treatment; D3: day 17 after end of antibiotic treatment; D4: day 35 after end of antibiotic treatment. E: Patient 10; E1: day -1 before start of treatment. E2: day 89 after start of treatment. E3: day 39 after end of antibiotic treatment. E4: day 75 after end of antibiotic treatment. F: Patient 12; F1: day 0, at start of treatment; F2: day 26 after start of treatment; F3: day 54 after start of treatment; F4: day 39 after end of antibiotic treatment. F5: day 72 after end of antibiotic treatment.

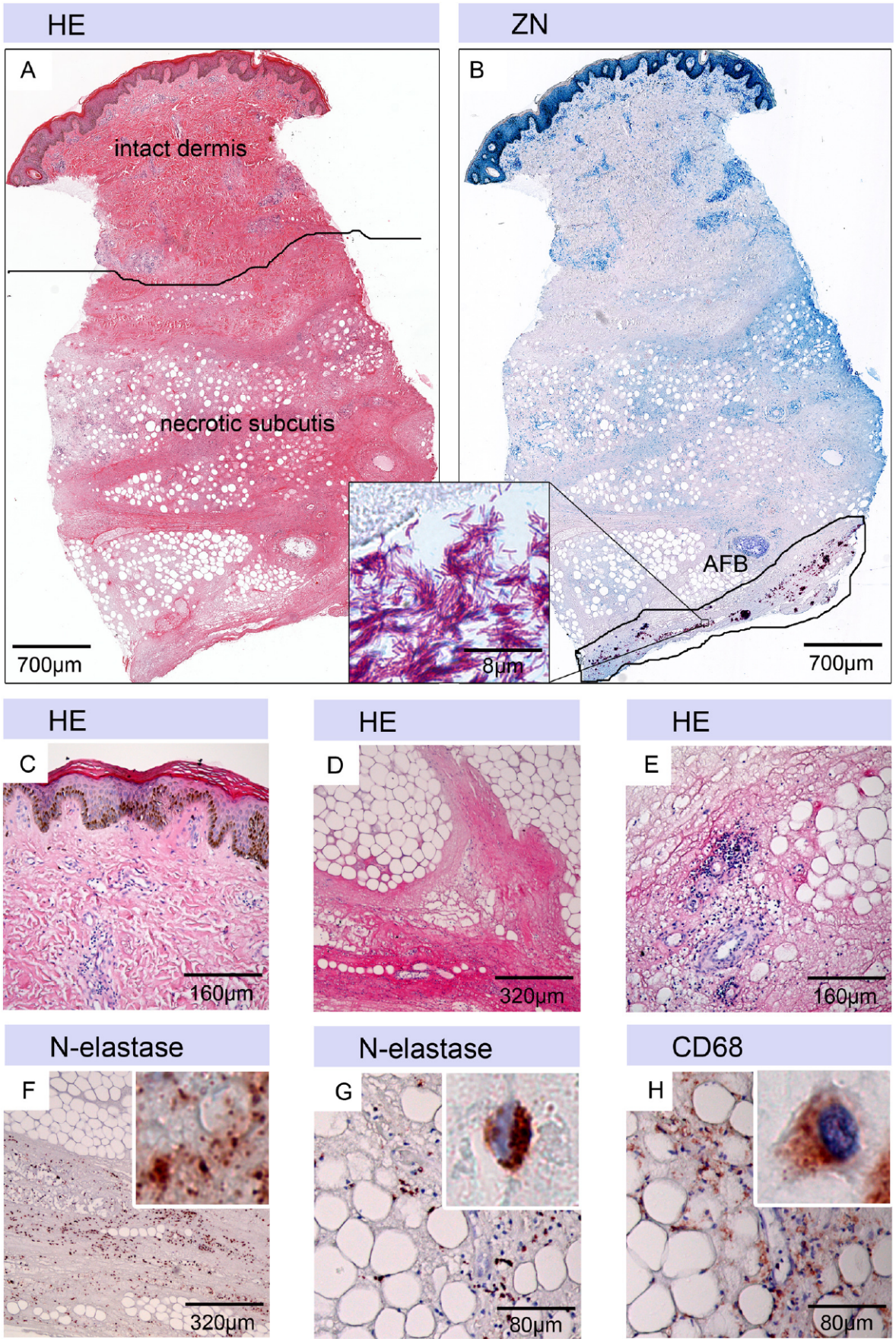

Figure 2: Characteristic histopathological features of tissue samples taken before start of antibiotic treatment

Histological sections were stained either with Haematoxylin-Eosin (HE) (A, C-E), Ziehl-Neelsen (counterstain methylenblue) (ZN) (B) or with antibodies against cell surface or cytoplasmic markers (counterstain haematoxylin) (F-H). A: Punch biopsy with large necrotic areas, fat cell ghosts and oedema but relatively intact epidermis and dermis. B: a band of extracellular AFBs is present in a deep layer of the necrotic subcutis. C: epidermis and dermis. D: necrotic region with fat cell ghosts. E: few infiltrating cells around a blood vessel. F: N-elastase staining revealed the presence of neutrophilic debris inside the necrotic regions. G: few intact neutrophils and H: CD68 positive infiltrating macrophages were found.

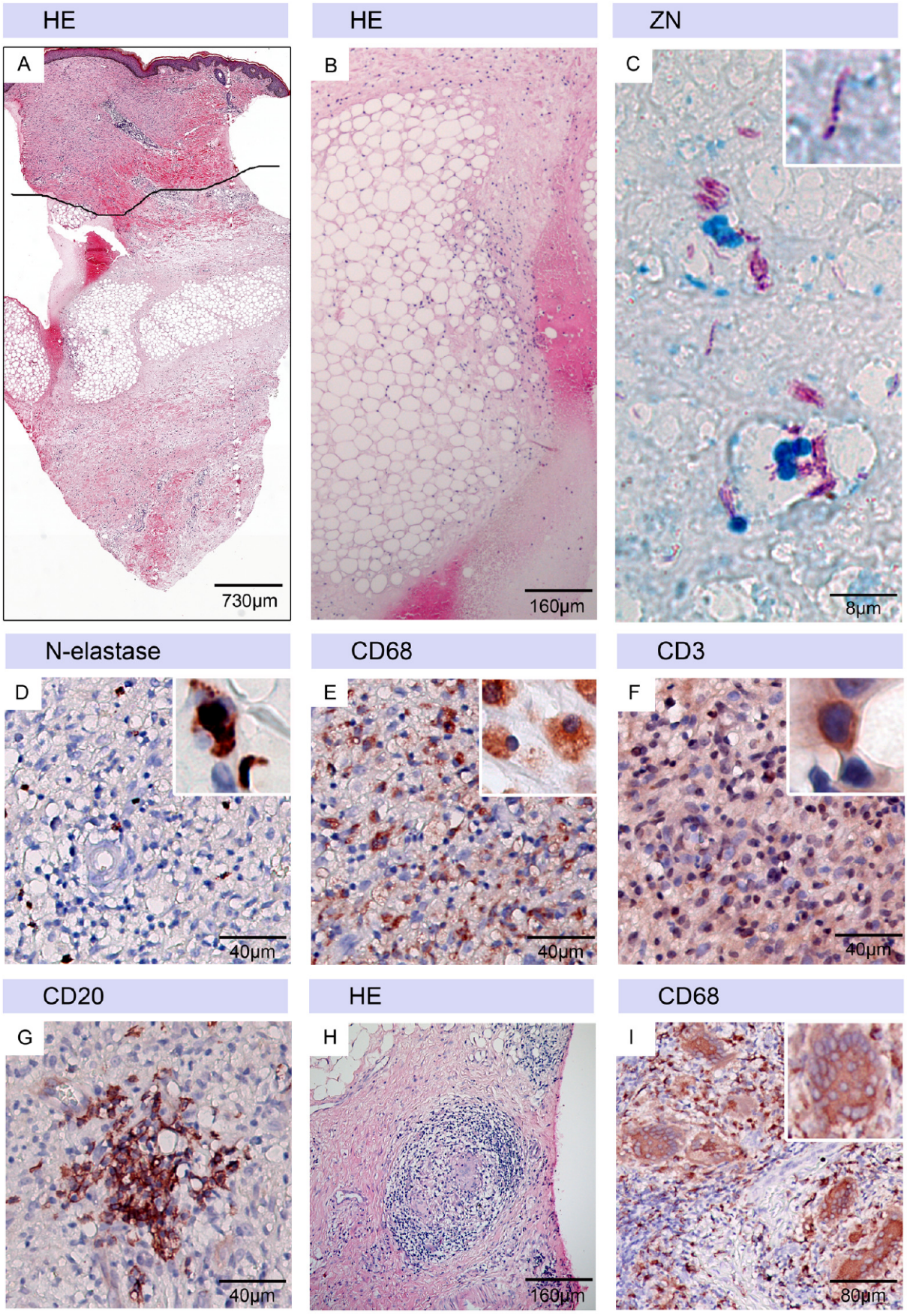

Figure 3: Characteristic histopathological features of tissue samples taken 26-34 days after start of antibiotic treatment

Histological sections were stained either with Haematoxylin-Eosin (HE) (A, B, H), Ziehl-Neelsen (counterstain methylenblue) (ZN) (C) or with antibodies against cell surface or cytoplasmic markers (counterstain haematoxylin) (D-G, I). A: Punch biopsy with large necrotic areas, fat cell ghosts and oedema but relatively intact epidermis and dermis. B: Higher magnification of necrotic tissue with large numbers of fat cell ghosts. C: Small numbers of intra and extracellular beaded AFB. D: N-elastase positive intact neutrophils were rare. E: More intact CD68 positive macrophages and F: CD3 positive T-cells were observed in the dermal tissue. Additionally, small CD20 positive B-cell cluster (G), few granulomas (H) and langhans giant cells (I) were found in only few of the samples.

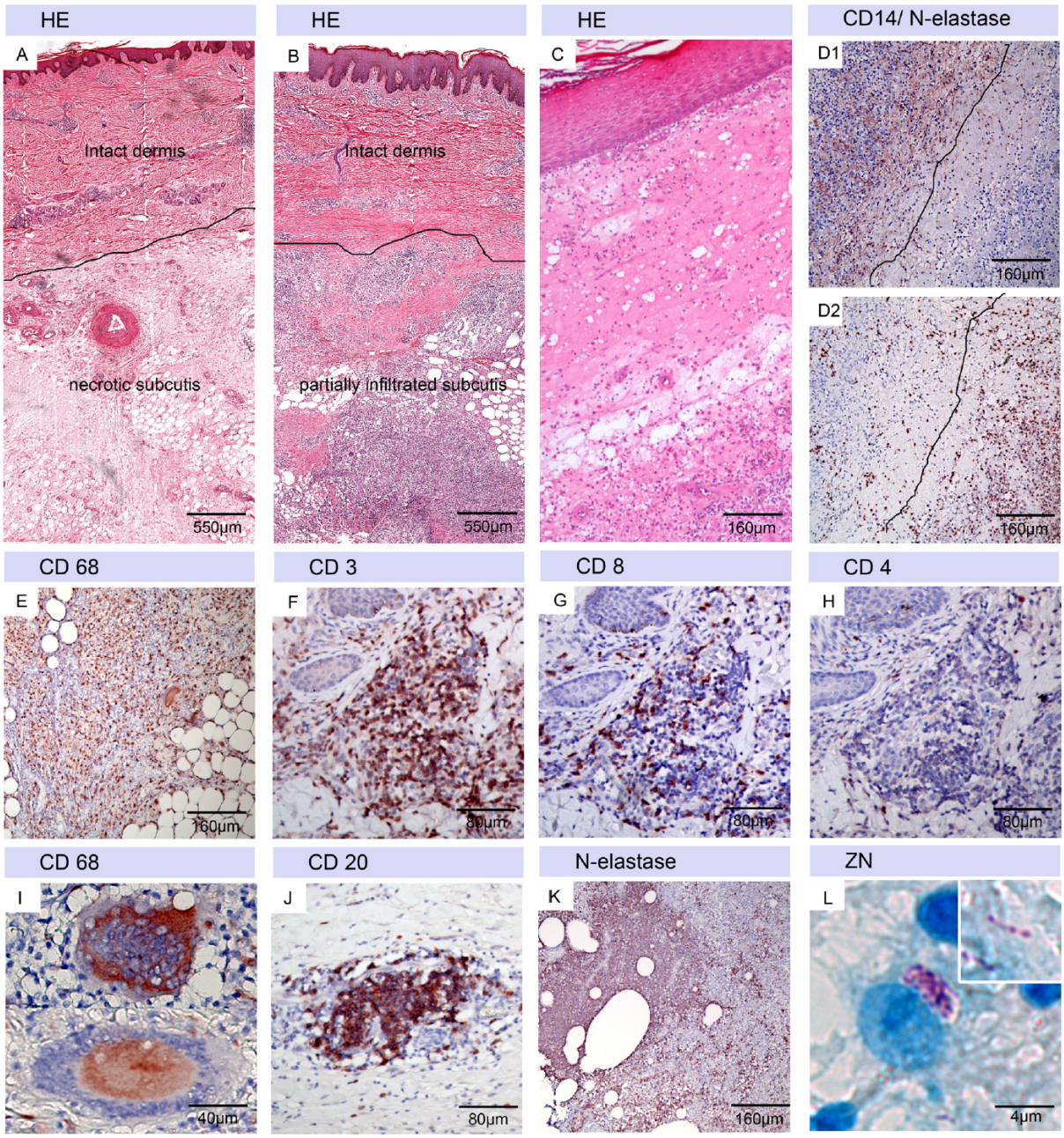

Figure 4: Characteristic histopathological features of tissue surgically excised to support wound healing

Histological sections were stained either with Haematoxylin-Eosin (HE) (A-C), Ziehl-Neelsen (counterstain methylenblue) (ZN) (L) or with antibodies against cell surface or cytoplasmic markers (counterstain haematoxylin) (D-K). A: Overview over an excised tissue specimen still harbouring large necrotic areas with fat cell ghosts and oedema. B: Overview over an excised tissue specimen presenting with mixed infiltration in the former necrotic region. C: Necrosis and oedema of the dermis of an excised non-ulcerative lesion. D: CD14 (D1) and N-elastase (D2) staining revealing a clear border between infiltration with intact CD14 positive macrophages (D1) and

neutrophilic debris inside the necrotic area (D2). Infiltrated tissue areas contained large numbers of CD68 positive macrophages (E) and large numbers of CD3 positive cells (F). These belonged mainly of the CD8 (G) and not of the CD4 (H) subset. Langhans and foreign body giant cells (I) and B-cell cluster (J) were present in the majority of the samples. Accumulations of N-elastase positive cells (K) were occasionally found. AFB were rare, had a beaded appearance and intracellular location (L).

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Chapter 5

Secondary Buruli Ulcer Skin Lesions Emerging Several Months after Completion of Chemotherapy: Paradoxical Reaction or Evidence for Immune Protection?

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Abstract

Background: The neglected tropical disease Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is an infection of the subcutaneous tissue leading to chronic ulcerative skin lesions. Histopathological features are progressive tissue necrosis, extracellular clusters of acid fast bacilli (AFB) and poor inflammatory responses at the site of infection. After the recommended eight weeks standard treatment with rifampicin and streptomycin, a reversal of the local immunosuppression caused by the macrolide toxin mycolactone of *M. ulcerans* is observed.

Methodology/ Principal findings: We have conducted a detailed histopathological and immunohistochemical analysis of tissue specimens from two patients developing multiple new skin lesions 12 to 409 days after completion of antibiotic treatment. Lesions exhibited characteristic histopathological hallmarks of Buruli ulcer and AFB with degenerated appearance were found in several of them. However, other than in active disease, lesions contained massive leukocyte infiltrates including large B-cell clusters, as typically found in cured lesions.

Conclusion/ Significance: Our histopathological findings demonstrate that the skin lesions emerging several months after completion of antibiotic treatment were associated with *M. ulcerans* infection. During antibiotic therapy of Buruli ulcer development of new skin lesions may be caused by immune response-mediated paradoxical reactions. These seem to be triggered by mycobacterial antigens and immunostimulators released from clinically unrecognized bacterial foci. However, in particular the lesions that appeared more than one year after completion of antibiotic treatment may have been associated with new infection foci resolved by immune responses primed by the successful treatment of the initial lesion.

Author Summary

Buruli ulcer (BU) is a chronic necrotizing skin disease presenting with extensive tissue destruction and local immunosuppression. Standard treatment recommended by the WHO includes 8 weeks of rifampicin/streptomycin and, if necessary, wound débridement and skin grafting. In some patients satellite lesions develop close to the primary lesion or occasionally also at distant sites during effective antibiotic treatment of the primary lesion. We performed a detailed analysis of tissue specimen from lesions that emerged in two BU patients from Benin 12 to 409 days after completion of chemotherapy. Histopathology revealed features of tissue destruction typically seen in BU and degenerated acid-fast bacilli. In addition, lesions contained organized immune infiltrates typically found in successfully treated BU lesions. Secondary lesions emerging many months after completion of chemotherapy may have been caused by immune response-mediated paradoxical reactions. The late onset may however also indicate that they were associated with new infection foci spontaneously resolved by adaptive immune responses primed by antibiotic treatment of the primary lesions.

Introduction

Buruli ulcer (BU) is a chronic necrotizing infection of subcutaneous tissue caused by *Mycobacterium ulcerans* [1-4]. BU seems to start usually as a movable subcutaneous nodule or papule and may later progress to a plaque or oedema. After destruction of subcutaneous tissue, the skin may break down centrally leading to the development of largely painless necrotic skin ulcers with characteristic undermined edges. These may progress to large necrotic lesions. *M. ulcerans* is unique among mycobacterial pathogens in that it resides in advanced lesions mainly extracellular. A histopathological hallmark of progressing BU is a poor local inflammatory response in the presence of clusters of extracellular acid-fast bacilli surrounded by areas of necrosis [5-7]. *M. ulcerans* produces a toxin with a polyketide-derived macrolide structure, named mycolactone, which plays a central role in tissue destruction and local immunosuppression. Observations both in cell culture and infection models indicate that cells infiltrating BU lesions are killed due to the cytotoxic and apoptosis inducing activity of mycolactone [7-10]. While *M. ulcerans* may be captured by phagocytes during initial stages of infection, it appears to persist only transiently inside these host cells [11,12]. After killing of the phagocytes, extracellular growth leads to the development of extracellular mycolactone-producing bacterial foci in areas of coagulating necrosis. Thermosensitivity of *M. ulcerans* seems to favour development of skin lesions of the limbs [13-15] .

Clinical diagnosis of BU can be confirmed by insertion sequence 2404 (*IS2404*) PCR [16-18], microscopic detection of acid-fast bacilli (AFB), culture of *M. ulcerans* [19] and histopathological examination of lesions [6,20-22]. While surgery has traditionally been the only recommended treatment for BU [23,24], WHO recommends currently as a first-line treatment a combination therapy with rifampicin and streptomycin (R/S) for eight weeks for all forms of the active disease [25,26]. After a pilot study assessing treatment of BU with R/S [25], a case-series in Benin showed that of 224 patients 215 were successfully treated [27], with 47% of them receiving antibiotics only. More recently, studies by Nienhuis et al., Kibadi et al. and Sarfo et al. [28-30] reconfirmed efficacy of R/S treatment. However, débridement, surgery and skin grafting may be used as an adjunct to the antimicrobial therapy, mainly to remove necrotic tissue, cover skin defects and correct deformities.

Reported rates of recurrence after surgical treatment alone range between 6% and 47% because even wide surgical excision of lesions may not remove all bacilli [31-34]. Recurrences may be caused by small numbers of *M. ulcerans* that have spread to healthy tissue surrounding the primary lesion [5]. Also lymphohematogenous spread of the mycobacteria may occur, since subsets of BU patients develop multiple skin lesions or metastatic osteomyelitis [35-39]. Although clinical trials indicate that some bacilli may survive the recommended eight week course of antibiotic treatment [28,30], recurrence rates after R/S treatment are as low as 1-2% [27,29].

In active BU disease, a protective cloud of mycolactone around the mycobacterial clusters is thought to both destroy infiltrating leukocytes and hinder them from passing pro-inflammatory signals to other cells. It is most likely, but still remains to be formally proven, that mycolactone production is reduced or abolished early after the onset of R/S chemotherapy due to impairment of mycolactone synthesis, bacterial growth arrest and/or bacterial cell death, reflected by ‘beaded’ appearance of AFBs (MT Ruf; unpublished results). Declining toxin levels allow leukocytes to reach the extracellular mycobacteria, leading to their phagocytosis and destruction [40]. Chronic leukocyte infiltration cumulates in the development of ectopic lymphoid structures [20]. After eight weeks of R/S chemotherapy, antigen presenting cells as well as B and T lymphocyte foci are found in large numbers inside the BU lesions [20] indicating that antigen recognition and processing is leading to active *M. ulcerans* specific immune responses. Vigorous local immune responses during R/S treatment may lead in some of the patients to the development of clinical deteriorations, ‘paradoxical reactions’ [41]. For this study we conducted detailed immunohistochemical analyses of secondary lesions which had occurred at extended periods of time after effective R/S treatment at different body sites.

Materials and Methods

Ethics statement

Ethical approval for analyzing patient specimens was obtained from the ethical review board of the Ministry of Health of Benin. Written informed consent from the guardians of the patients was obtained before surgical specimens were used for reconfirmation of BU as well as a detailed immunohistological analysis.

Study participants

Both patients, two six year old boys, included in this study were laboratory-confirmed BU cases with one primary lesion. Both received a combination of rifampicin (10mg/kg body weight) and streptomycin (15mg/kg body weight) administered daily over 8 weeks at the Centre de Diagnostic et de Traitement de l'Ulcère de Buruli (CDTUB) in Pobè, Benin according to the WHO recommendations. Both patients developed several new lesions at different parts of the body, 12 - 409 days after completion of antibiotic treatment. These lesions were removed by limited excision and no additional antibiotic treatment was administered. Excised tissue from a number of these new lesions became available for histopathological analysis (Table 1). Both patients were tested negative for HIV, shistosomiasis, hepatitis B and syphilis. Blood values tested and the nutritional status was within the limits typically found in children in rural Africa. Only patient 2 presented with a BCG scar.

Histopathological analysis

Tissue specimens analyzed are listed in Table 1. Samples were fixed in 4% neutral-buffered paraformaldehyde for 24 h and subsequently transferred to 70% ethanol for storage and transport. Afterwards biopsies were dehydrated, embedded in paraffin, and cut into 5 µm thin sections. After deparaffinization and rehydration sections were either directly stained with haematoxylin/eosin (HE) or Ziehl-Neelsen/methylenblue (ZN) according to WHO standard protocols [42] or further processed for immunohistochemistry (IHC). For IHC antigen retrieval was performed according to standard protocols either with citrate buffer, EDTA buffer or by enzymatic trypsin digestion (Dako® Education guide: Immunohistochemical Staining methods). Afterwards endogenous peroxidase was inactivated with 0.3% H_20_2 for 20min and prevention of unspecific binding was achieved by incubation with blocking serum

matching the secondary antibody host. Primary antibodies specific for N-Elastase (polymorphonuclear neutrophils [PMNs]; Dako clone NP57), CD3 (T-lymphocytes; Dako clone F7.2.38), CD8 ($CD8^+$ T-lymphocytes; Serotec clone 4B11), CD14 (Monocytes/macrophages; Novocastra clone 7) and CD20 (B-lymphocytes; Novocastra clone7D1) were appropriately diluted in phosphate buffered saline (PBS) containing 0.1% Tween-20 and added to the slides for 1 h at room temperature or over night at 4°C. After incubation with a matching biotin-conjugated secondary antibody staining was performed using the Vector NovaRED system. Haematoxylin was used as a counter stain.

Results

Clinical presentation of BU patients developing secondary skin lesions after completion of antibiotic treatment

In the present report we describe clinical and histopathological observations in two BU patients that have developed series of new skin lesions (Table 1) after effective anti-mycobacterial chemotherapy.

Patient 1, a six year old boy, presented at the Centre de Diagnostic et de Traitement de l'Ulcère de Buruli (CDTUB) in Pobè, Benin with a 15x15cm ulcerated plaque lesion at the right forearm and elbow with undermined edges characteristic for BU (Figure 1A). First BU symptoms had been noticed eight month before and the lesion had been treated afterwards with traditional medication. After admission to the hospital clinical diagnosis was confirmed by a positive *IS2404* PCR result of a fine needle aspirate, whereas culture was negative. As recommended in the WHO guidance on the role of specific antibiotics in the management of BU [26] the patient received for 8 weeks daily oral rifampicin (10mg/kg body weight) and intramuscular streptomycin (15mg/kg body weight). 37 and 65 days after start of this standard R/S chemotherapy wound débridement was performed, 18 days after the last excision skin grafting was done and 83 days after grafting the primary lesion had healed.
75 days after completion of chemotherapy a first new ulceration 0,5x0,5 cm (ulcer 1) in the axilla of the right arm emerged. After performing some débridement, this lesion had healed 35 days later and the patient was discharged from hospital. 275 days after completion of chemotherapy the patient was readmitted with a non ulcerated fluctuant nodule 1,5x1,5 cm (nodule 1) on the back (Figure 1B), which was excised with primary skin closure one day later. 409 days after completion of chemotherapy two more lesions developed, a 1,5x1,5 cm nodule (nodule 2) on the thorax (Figure 1C) and an ulcerated plaque 3x3cm on the right shoulder (ulcer 2) (Figure 1D). Both lesions were excised two days after admission. From both lesions specimen taken were *IS2404* PCR as well as AFB positive, whereas culture was negative. 28 days after the surgical intervention, the patient was discharged from hospital. No further relapses were observed after 10 months of follow-up (February 2011).

Patient 2, also a six year old boy, presented at the CDTUB with a 20x15cm ulcerated lesion on the interior side of his right upper leg and knee. Undermined edges as well as 'cotton wool' appearance of necrotic tissue at the center of the lesion were characteristic for BU [42]. Clinical diagnosis was confirmed by positive *IS2404* PCR results and microscopic detection of AFB in swab samples. Surgical débridement was performed 29 days after start of standard R/S chemotherapy followed 10 days later by skin grafting. Twelve days after completion of antibiotic treatment, a nodule (nodule 1) 2x2 cm; had emerged about 5 cm proximal of the border of the primary lesion at the upper right leg and was excised 7 days later. The initial lesion as well as the lesion at the excision site had healed 39 days after completion of the antibiotic treatment (i. e 57 days after skin grafting) and the patient was discharged from hospital.

One week after discharge (46 days after completion of antibiotic treatment) the patient was readmitted with a second nodule (1,5x1,5 cm) located at the lower right leg about 15 cm distal of the border of the primary lesion. Again eight days later (54 days after completion of antibiotic treatment) a third nodule (nodule 3) (3x2 cm) had emerged at the upper right leg located 5cm proximal of the initial wound. These two nodules were excised 93 days after completion of the antibiotic therapy. While AFB staining, as well as *IS2404* PCR confirmed the presence of *M. ulcerans,* both nodules were culture negative.

After surgical excision and healing of the satellite lesions the patient was discharged, but re-admitted 176 days after completion of antibiotic treatment with a fourth nodule (nodule 4) 2x2cm on the right foot. A minimal surgical intervention was performed and the patient was discharged 10 days later and no further relapses were observed after 10 months of follow-up (February 2011).

Histopathological features of excised secondary lesions

Histopathological and immunohistochemical analyses were performed with nodule 1, nodule 2 and ulcer 2 from patient 1, and nodule 2 and 3 of patient 2 (Table 1). These lesions appeared 275 to 409 days and 46 to 54 days, respectively, after completion of chemotherapy. Analysis yielded comparable results for all specimens analyzed. Typical data are shown below.

Features characteristic for BU pathology, such as fat cell ghosts, necrotic soft tissue, haemorrhages, and epidermal hyperplasia were present in all specimens analyzed.

As shown in Figure 2A, necrotic areas were massively infiltrated with leucocytes, a feature, which is characteristic for successfully treated lesions [20]. Immunohistochemical analysis revealed mixed cellular infiltrations (Region 1) composed of large numbers of CD14 positive macrophages/monocytes (Figure 2B) and CD3 positive T-cells (Figure 2C). In contrast, intact N-elastase positive neutrophils were rare (Figure 2D). As described previously [20], some areas, such as the AFB containing region 2 in Figure 2A contained N-elastase positive debris (Figure 2E), which appears to represent remains of an early wave of neutrophilic infiltration. ZN staining revealed AFBs and globi like structures in the necrotic areas in the tissue from nodule 1 of patient 1 and in nodules 2 and 3 of patient 2 (Figure 2F, G). AFBs had a 'beaded' appearance (Figure 2G), which has been shown to be an indicator for loss of viability in the case of *M. leprae* [43].
Clusters of CD20 positive B-cells, another hallmark of ectopic lymphoid tissue developing in BU lesions after successful treatment [20], were also found in the tissue specimens analyzed. These clusters varied in size ranging from very large accumulations forming a band throughout the whole tissue (Figure 3A) to small dense B cell accumulations (Figure 3B) surrounded by CD14 positive macrophages/monocytes (Figure 3C) and few interspersed CD3 positive T-cells (Figure 3D), mostly CD8 negative (Figure 3E), mainly at the border of the dense B-cell cluster. Higher magnifications confirmed the dense packaging of B-cells (Figure 3F) and more dispersed distribution of other leucocytes (Figure 3G-H).

In some parts of the lesions small uninfiltrated necrotic areas surrounded by belts of leucocytes still remained. Immunohistochemical analyses gave indications for sequential infiltration of these areas by different types of leucocytes (Figure 4). Necrotic areas were surrounded by an inner dense belt of CD14 positive macrophages/monocytes (Figure 4A), which thus seem to constitute the first line of infiltration after decline of cytotoxic mycolactone levels. While a belt containing large numbers of CD3 positive T-cells representing a second line of infiltration were found in direct neighbourhood to the macrophages (Figure 4B), intact N-elastase positive neutrophils (Figure 4C) and CD20 positive B-cells (Figure 4D) were comparatively rare in these settings. However a strong staining of N-elastase positive neutrophilic debris was observed inside the necrotic areas (Figure 4C). Higher magnification revealed no intact cells in this location (Figure 4C insert).

Discussion

In this report we describe the development of series of new skin lesions in two BU patients 12 – 409 days after completion of antibiotic treatment. The newly emerging nodules and ulcerations were located either at some distance from the initial lesion at the same extremity or at other body locations. Detection of *M. ulcerans* DNA by *IS2404* PCR, microscopic detection of AFBs and the presence of histopathological features characteristic for BU demonstrated that the new lesions were associated with *M. ulcerans* infection. Degenerated appearance of the AFBs and the presence of massive immune cell infiltrates in most parts of the lesions were on the other hand characteristic for treated BU lesions [20].

Detailed immunohistochemical analyses showed that residual necrotic areas were surrounded by an outer belt of T-lymphocytes and an inner belt of macrophages/monocytes with appendices reaching into the necrotic tissue. These belts of intact leucocytes seem to reflect ongoing efforts of the immune system to resolve the necrotic areas. In contrast, remains of neutrophils found inside the necrotic areas seem to be leftovers of early acute neutrophilic infiltration waves. These are also observed in early phases of *M. ulcerans* infection in experimentally infected mice (MT Ruf et al., unpublished results). Apart from these residual necrotic regions, the destroyed adipose and dermal connective tissue layers showed angiogenesis and contained abundant leukocyte infiltrates. It is thought that such chronic infiltrates can only develop once the concentration of the cytopathic *M. ulcerans* macrolide toxin mycolactone has diminished [20,44]. Imbedded in the diffuse infiltrates, more structured leukocyte accumulations, such as B-cell clusters indicative for humoral immune responses [45-47] and first granulomas were found. In BU granulomas may function primarily as a place for antigen presentation and adaptive immune response, rather than for sequestration of the mycobacteria [20].

Recently O'Brien et al have described the occurrence of paradoxical reaction in two Australian BU patients during R/S treatment of BU [41]. After a first clinical improvement worsening of the clinical appearance occurred. For one patient incomplete excised wound margins showed paradoxical reaction whereas for the other patient a more distant secondary lesion opened, before end of treatment was

reached. Worsening of lesions motivated a change in the treatment regimen and additional surgery. After detailed evaluation, data have been interpreted as immune-mediated reactions rather than treatment failures, as it has been shown that antibiotic therapy for *M. ulcerans* leads to a reversal of local immunosuppression [20,41,48]. The observed vigorous local immune responses are most likely caused by bacterial antigens and immunostimulators released from the killed mycobacteria. Similar paradoxical reactions have been well described for *M. tuberculosis*, *M. leprae* and in particular in immunocompromised HIV patients who commence HAART [49-52]. In tuberculosis an elevation of the TNF‐α level, stimulated by lipoarabinomanan and other lipopolysaccharides present in the cell wall, has been postulated as an initial step in the development of paradoxical reaction [53,54]. Limited surgical excision may help to resolve paradoxical reactions by reducing the burden of mycobacterial antigens and in some clinical settings corticosteroids have been used for down regulation of immune responses [55-58].

In the case of the two patients described here, new lesions developed at prolonged periods of time after completion of antibiotic treatment. These lesions may represent secondary *M. ulcerans* infection foci that were already present without clinical signs and symptoms during antibiotic treatment and development of new lesions may be the consequence of delayed paradoxical reactions. However, in particular the lesions that appeared more than one year after completion of antibiotic treatment may also have been associated with new infection foci caused by new *M. ulcerans* infections or by mycobacteria that had survived the eight week course of R/S treatment [28,29]. These may have been resolved by immune responses primed by the successful treatment of the primary lesion. If this is the case, detailed analysis of immune responses in more patients developing such secondary lesions may provide important insights into immune protection against *M. ulcerans* and support vaccine design [59].

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Table

Table 1: Features of skin lesions that emerged after completion of antibiotic treatment. Lesions from which tissue samples became available for histopathological analysis are written in bold letters.

Table 1. Features of skin lesions that emerged after completion of antibiotic treatment.

Patient	Primary lesion	Time span (days) between completion of antibiotic treatment and occurrence of secondary lesion	Time span (days) between occurrence of secondary lesion and surgical excision	Nature of satellite lesion	Location of satellite lesion	Distance of satellite from primary lesion
1	ulcer at the right upper arm/elbow	75	4	ulcer 1	right axilla	10 cm
		275	1	**nodule 1**	back	25 cm
		409	2	**ulcer 2**	right shoulder	20 cm
		409	2	**nodule 2**	thorax	30 cm
2	ulcer at the right upper leg/knee	12	6	nodule 1	right upper leg	5 cm
		46	33	**nodule 2**	right lower leg	15 cm
		54	25	**nodule 3**	right upper leg	5 cm
		176	1	nodule 4	right foot	30 cm

Lesions from which tissue samples became available for histopathological analysis are written in bold letters.
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Figures

Figure 1: Clinical presentation of lesions (Patient 1)

A: Initial ulcerated lesion at the right arm, reaching from the elbow to the forearm. B: Nodule1 appearing on the back, 275 days after end of antibiotic treatment. Both, nodule 2 on the thorax (C) and an ulcerated plaque on the right shoulder (D) had appeared 409 days after completion of antibiotic treatment.

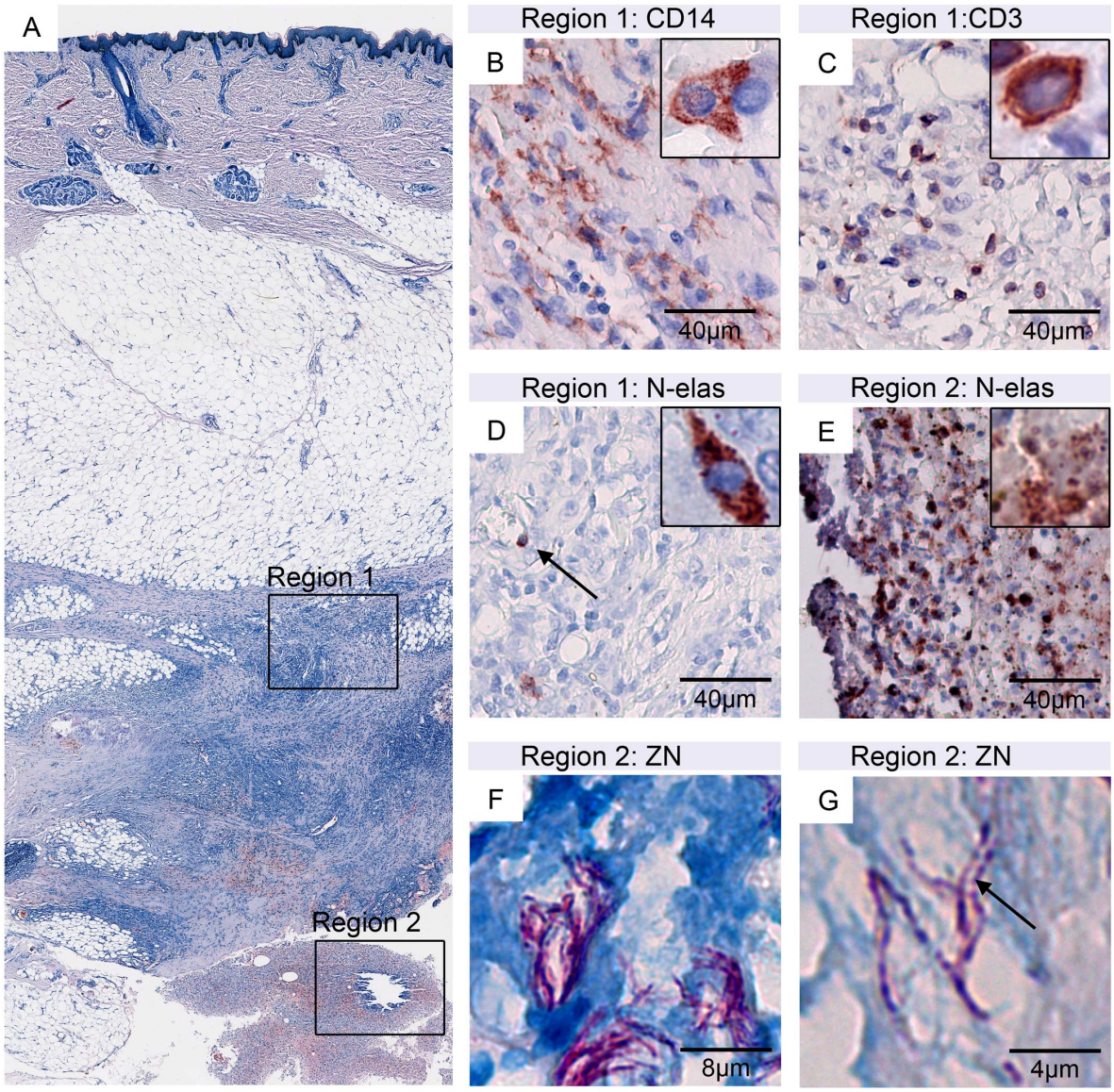

Figure 2: Histopathological presentation of secondary lesions

Histological sections (nodule 2 of patient 2) were stained either with Ziehl-Neelsen (counterstain methylenblue; A, F, G) or with antibodies against cell surface or cytoplasmic markers (counterstain haematoxylin; B-E). A: Overview over excised tissue specimen revealing typical BU pathology features like fat cell ghosts, necrosis, epidermal hyperplasia and AFB (region 2) as well as a strong mixed infiltration typically observed in successfully treated BU lesions (region 1). B: CD14 staining of macrophages/monocytes; C: CD3 staining of T-cells; D: Elastase staining of neutrophils. In the necrotic region 2 large numbers of elastase-positive neutrophilic debris (E) and small clumps of AFB (F) with a beaded appearance (G) were observed.

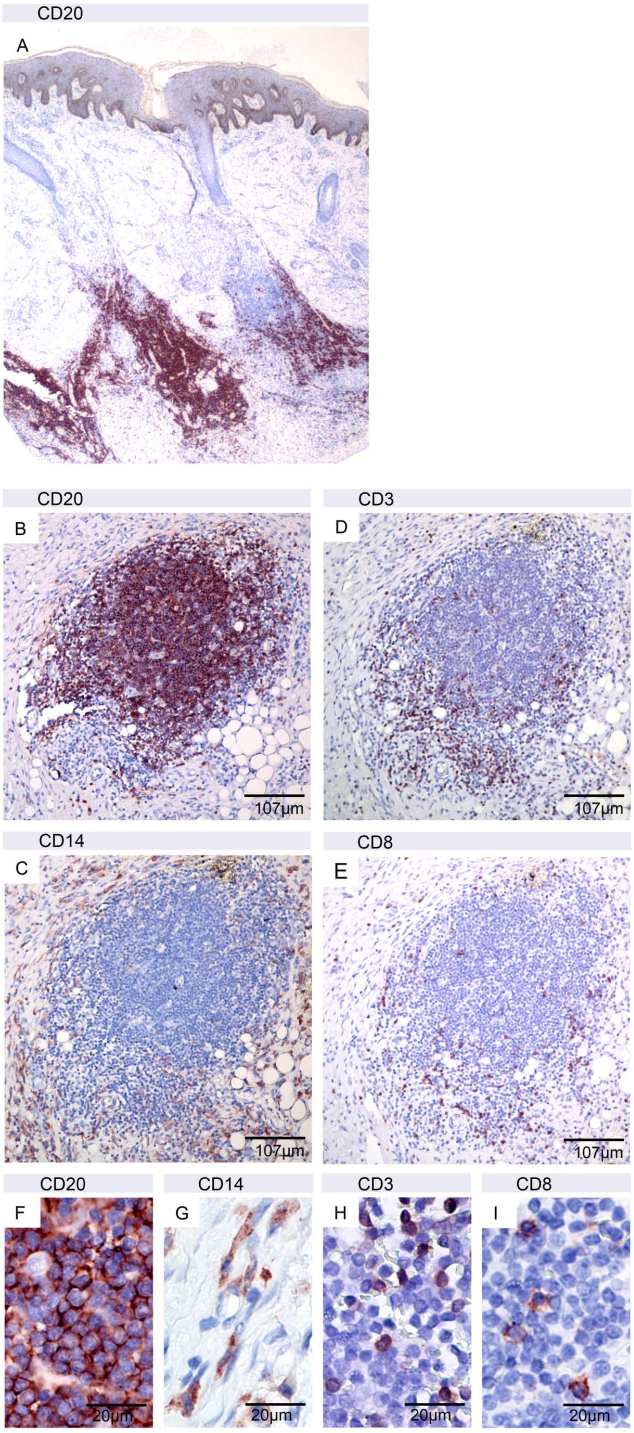

Figure 3: Presence of B-cell clusters in the secondary lesions

A: Band of CD20 positive B-cells in sections of ulcer 2 of patient 1. B-E: serial sections of nodule 3 of patient 2 with a small dense cluster of CD20 positive B-cells (B) surrounded by CD14 positive macrophages/monocytes (C) and few interspersed CD3 positive T-cells (D) from which the majority was CD8 negative (E). Higher magnification (F-I) revealed a very dense package of the B-cells.

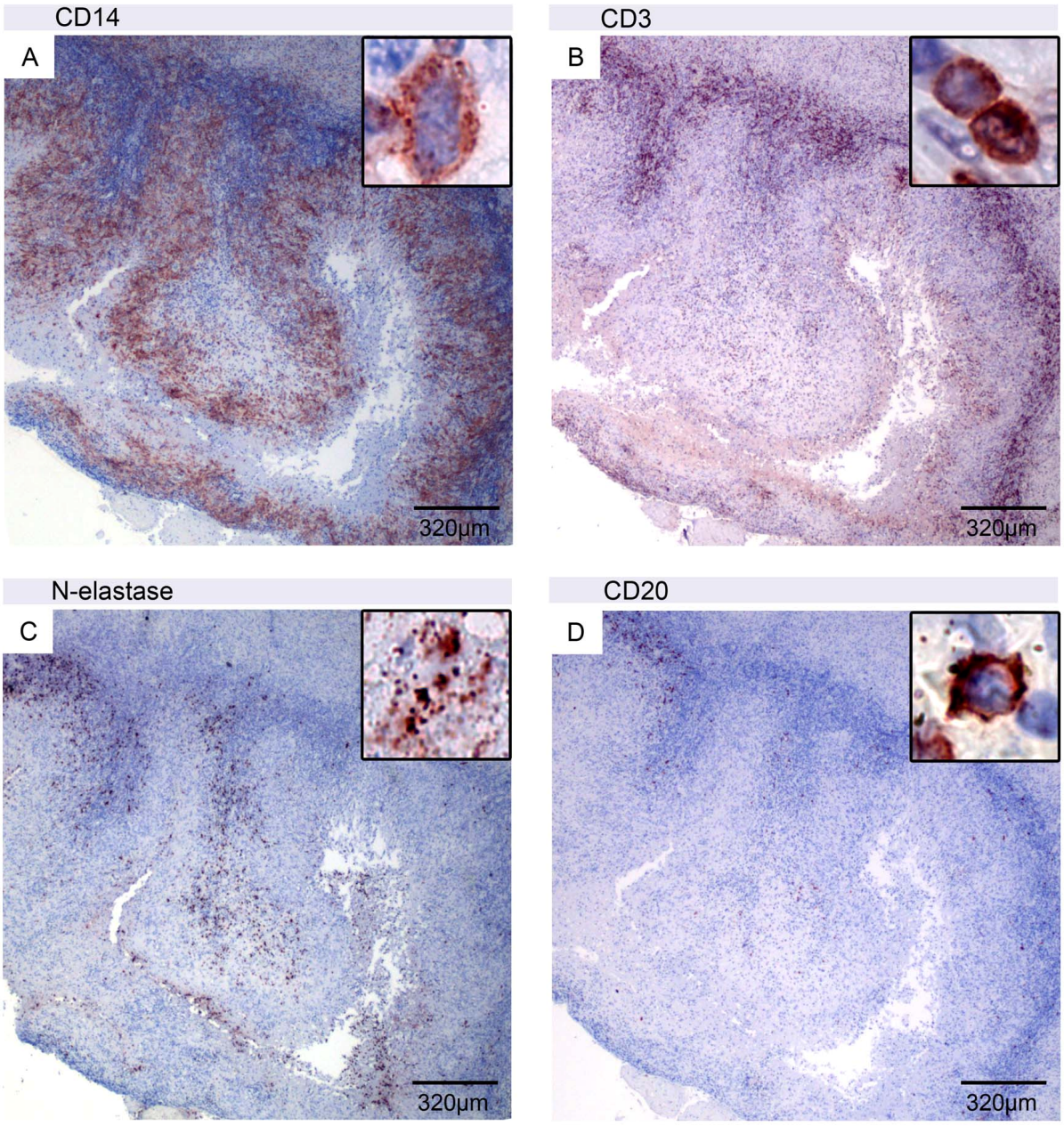

Figure 4: Bands of leucocytes surrounding an uninfiltrated necrotic area

Serial sections of nodule 2 of patient 1 with a necrotic area surrounded by a belt of CD14 positive monocytes/macrophages (A) and a more external second belt of CD3 positive T-cells (B). The necrotic core contained N-elastase positive neutrophilic debris (C), but no intact neutrophils (D insert). Clusters of CD20 positive B-cells were found away from the necrotic core (D).

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Chapter 6

Oral Treatment for *Mycobacterium ulcerans* Infection: Results from a Pilot Study in Benin

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Abstract

Mycobacterium ulcerans infection is responsible for severe skin lesions in sub-Saharan Africa. We enrolled 30 Beninese patients with Buruli ulcers in a pilot study to evaluate efficacy of an oral chemotherapy using rifampicin plus clarithromycin during an 8-week period. The treatment was well tolerated, and all patients were healed by 12 months after initiation of therapy without relapse.

Introduction

Mycobacterium ulcerans is the causative agent of Buruli ulcer, an emerging tropical disease marked by devastating skin lesions [1]. Buruli ulcer affects mainly children in rural areas, where access to health care is often delayed and where lengthy hospital stays are problematic. Until recently, surgery was the only treatment for Buruli ulcer. Significant progress has been made in the past 5 years with the demonstration of the efficacy of rifampicin plus streptomycin (R + S) chemotherapy [2]. Its routine implementation has dramatically improved healing while reducing the frequency of relapses [3]. However, streptomycin is an injectable drug, and the lack of an efficacious oral treatment remains one of the main obstacles to decentralizing care at local level.

A recently published randomized controlled trial from Ghana showed no significant difference in the proportion of patients who achieved cure after receiving the World Health Organization (WHO)–recommended 8-week course of R + S chemotherapy, compared with the proportion who achieved cure after receiving a treatment consisting of 4 weeks of R + S followed by 4 weeks of rifampicin + clarithromycin (R + C) [4].

This study represents a significant step to improve Buruli ulcer treatment. Indeed, clarithromycin is orally administered and is better tolerated than streptomycin, which requires daily injections and is associated with adverse events, such as vestibular toxicity (which occurs in 0.5%–5% of patients) and nephrotoxicity (which occurs in 5%–10%) [5]. A clarithromycin-based oral treatment would be more easily administered, better accepted by patients, and contribute to limiting the number of injections in the developing world [6]. Implementation of an oral R + C chemotherapy is supported by recent evidence of its bactericidal activity in vivo [7, 8] and by several clinical successes [9, 10].

Here, we report a pilot study involving 30 patients with Buruli ulcer disease treated using an oral combination of rifampicin and clarithromycin over an 8-week period. All patients were successfully healed, and no adverse event was observed.

Materials and Methods

This study was realized at the Centre de Diagnostic et de Traitement de l'Ulcère de Buruli (CDTUB) in Pobè, Benin. Eligible patients had laboratory-confirmed cases of Buruli ulcer, were at least 5 years of age, presented with lesions ≤10 cm in diameter that had appeared within the past 6 months, agreed to be hospitalized during treatment, and were likely to be followed up for 18 months (i.e., had stable habitation). Written informed consent was obtained from the patient and from the patient's parent or guardian if the patient was under 18 years of age.

Noninclusion criteria were lesions >10 cm in diameter, multiple lesions, lesions located over a joint, history of treatment with antimycobacterial drugs, receipt of macrolide or quinolone antibiotics during the previous month, allergy to rifampicin or clarithromycin, pregnancy, or human immunodeficiency virus infection. All nonincluded patients were treated according to WHO guidelines.

The primary end point was defined as healing of the wound at 12 months, without recurrence 18 months after initiation of chemotherapy. Patients were treated using an oral combination of rifampicin (10 mg/kg) and clarithromycin (12 mg/kg) administered simultaneously, once daily, over 8 weeks.

During treatment, patients were hospitalized and attended daily by nursing staff, who dispensed chemotherapy with a fatty snack and monitored patients for adverse effects during a 1-h period after treatment intake. Clarithromycin was administered as 250-mg tablets completed with syrup to achieve exact dose. Nursing staff cleansed the wound using physiological solution and renewed the wound dressing using simple sterile dressings. Every week, patients were examined by a doctor, who also collected samples at week 4, 6, and 8 if the lesion had not healed, using swabs or aspiration. These samples were sent for *M. ulcerans* culture and polymerase chain reaction (PCR) analysis. After the wound closed, patients were discharged from the hospital and were followed-up every 3 months up to 18 months after starting treatment.

Limited surgery was defined as curettage of the lesion or a minor excision to remove excess granulation tissue and to debride ulcer margins. Extensive surgery was defined as major excision followed by skin-grafting. Surgery was undertaken if: (1) no improvement or an aggravation of the lesion characteristics was observed at week 4 of chemotherapy or after, (2) induration persisted 12 weeks after initiation of

chemotherapy, (3) the lesion started bleeding at any time, or (4) the lesion showed signs of improper scaring and risks of functional incapacities at any time. Excised tissue was sent for histological analysis, as well as *M. ulcerans* culture and PCR.

All biological samples were collected and handled identically according to WHO guidelines. Blinded analysis of samples was performed at the laboratory in Angers, France, along with routine microbiological analyses for the CDTUB.

Ethical approval for this study was given by the Ministry of Health in Benin. Technical approval was given by the WHO Buruli ulcer working group on chemotherapy.

Results

Patients were enrolled consecutively from December 2007 through February 2009. Included patients represented one-third of eligible patients who received a diagnosis during this period. The main reason for nonparticipation was refusal of hospitalization. Twelve of the patients were male, and 18 were female; 11 were >15 years of age. Nine patients presented with nonulcerative lesions, and 21 presented with ulcerative lesions. Buruli ulcer diagnosis was confirmed by PCR for all patients, and 13 patients (43%) had a positive culture result at enrolment.

Treatment was well tolerated, and no adverse events were reported. All patients had reached 18 months of follow-up by September 2010.

All 30 patients were successfully treated, with complete re-epithelialization of wounds 12 months after treatment initiation, and no patient experienced relapse during follow-up. The median duration of healing was 104 days (range, 30–212 days; see Table 1 and supplementary Table 1 for individual data).

Microbiological follow-up was performed at week 4, 6, and 8 on lesions that remained open. Culture results became negative for all sampled patients except 1 after 4 weeks; no culture was positive after 6 weeks. PCR follow-up of lesions that remained open showed that negative PCR results were rarely obtained before scarring (supplementary Table 2).

Among those 30 patients, 15 (50%) healed after chemotherapy without any additional intervention (Table 1), and 11 (37%) of the patients underwent limited surgical procedures, such as curettage (n = 9) or excision (n = 2). These procedures were mainly undertaken without suspicion of failure to promote a faster and more regular scaring of lesions presenting excessive granulation or risks of functional incapacities.

Four patients, 6–8 years of age, underwent extensive surgical excision followed by skin grafting. Three patients presented with category 2 lesions that were large for their body size and either worsened or showed no improvement (supplementary Table 1). One patient who had previously healed without surgery experienced trauma at the site of the scar and required secondary extensive surgery.

As observed in our routine experience of R + S chemotherapy [3], R + C chemotherapy was also sufficient to cure most (8 of 10) patients with category 1 ulcerative lesions and to cure 5 of 11 patients who presented with larger ulcerative

lesions. A majority of nonulcerative lesions (7 of 9) required additional surgical procedures.

Tissue specimens were collected from the 15 patients who underwent surgery. Of 14 samples analyzed in culture, none was positive for *M. ulcerans*. However, 13 patients had a positive PCR result, suggesting persistence of mycobacterial material, as described by others [2].

Discussion

In this pilot study, 30 patients with confirmed Buruli ulcer disease were successfully healed using R+C chemotherapy. Microbiological results indicate that persistence of viable mycobacteria was unlikely, even when extensive surgery was required. Histological analysis supports this point, showing that 10 of 11 samples analyzed presented evidence of massive inflammatory infiltration. Local inflammatory reactions and the development of ectopic lymphoid tissue have been observed in Buruli ulcer lesions during healing while receiving chemotherapy [11, 12]. This most likely results from mycobacterial antigens and immunostimulators released during chemotherapy. In our experience, as confirmed by others [13], this reaction is associated with good response to treatment and occurs irrespective of the chemotherapy used (A.C., unpublished data).

We advocate that additional chemotherapy efficacy studies should rely on an improved definition of treatment failure that includes microbiological evidence of viable, treatment-resistant mycobacteria and histological assessment of inflammatory reaction. Clinical criteria do not provide a clear rule to discriminate between patients who experience chemotherapy failure and patients who successfully clear the infection but require surgery to expedite the healing process.

In the Ghanaian trial, treatment failure was defined according to clinical criteria, such as lesion size progression or the need for extensive surgery, for all but one of the patients who experienced treatment failure [4]. The subjective component of such clinical criteria makes it difficult to compare results across different studies. In our study, 3 patients required extensive surgery, but none presented persistence of viable mycobacteria.

Positive cultures were obtained in Ghana after treatment completion in 5 patients who belonged to the 4 weeks of R + S followed by 4 weeks of R + C arm [4]. This result is difficult to interpret without information on drug resistance or possible re-infections. In our study, viable mycobacteria were not observed after 6 weeks of treatment. We hypothesize that our higher dosage of clarithromycin was more efficient at clearing the infection, without causing more adverse effects.

In conclusion, this study provides compelling evidence to support a future randomized controlled trial that compares the standard regimen (8 weeks of R + S)

with the complete oral regimen (8 weeks of R + C). Proving the efficacy of the R + C combination could lead to a simpler, less invasive, and less painful treatment that is easier to implement at the local level. Surgery is likely to remain necessary for severe lesions, but a large proportion of patients can be cured through oral chemotherapy alone, which is a great boon to the rural communities most affected by Buruli ulcer.

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Table

Table 1: Outcome and Additional Care received in 30 Patients with Buruli Ulcer Disease Treated with Oral Rifampicin plus Clarithromycin (R + C) Chemotherapy over an 8-Week Period

Lesion type	World Health Organization Category[a]	No. (%) of patients successfully healed at 12 months				Median time to healing (days)
		Without surgery	With limited surgery	With excision and skin-grafting	Total	
Ulcerative	1	8	2	0	10	38
	2	5	4	2*	11	115
Non ulcerative	1	2	1	0	3	109
	2	0	4	2	6	111
Total		15 (50)	11 (37)	4* (13)	30 (100)	104

[a] Category 1, lesion diameter <5 cm; category 2, lesion diameter 5–10 cm.

* Including one patient healed with chemotherapy alone who required secondary skin-grafting after experiencing trauma on the scar site.

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Chapter 7

Independent Loss of Immunogenic Proteins in *Mycobacterium ulcerans* Suggests Immune Evasion

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Abstract

The highly immunogenic mycobacterial proteins ESAT-6, CFP-10, and HspX represent potential target antigens for the development of subunit vaccines and immunodiagnostic tests. Recently, the complete genome sequence revealed the absence of these coding sequences in *Mycobacterium ulcerans*, the causative agent of the emerging human disease Buruli ulcer. Genome reduction and the acquisition of a cytopathic and immunosuppressive macrolide toxin plasmid are regarded as crucial for the emergence of this pathogen from its environmental progenitor, *Mycobacterium marinum*. Earlier, we have shown the evolution of *M. ulcerans* into two distinct lineages. Here, we show that while the genome of *M. marinum* M contains two copies of the *esxB-esxA* gene cluster at different loci (designated MURD4 and MURD152), both copies are deleted from the genome of *M. ulcerans* strains belonging to the classical lineage. Members of the ancestral lineage instead retained some but disrupted most functional MURD4 or MURD152 copies, either by newly identified genomic insertion-deletion events or by conversions of functional genes to pseudogenes via point mutations. Thus, the *esxA* (ESAT-6), *esxB* (CFP-10), and *hspX* genes are located in hot-spot regions for genomic variation where functional disruption seems to be favoured by selection pressure. Our detailed genomic analyses have identified a variety of independent genomic changes that have led to the loss of expression of functional ESAT-6, CFP-10, and HspX proteins. Loss of these immunodominant proteins helps the bacteria bypass the host's immunological response and may represent part of an ongoing adaptation of *M. ulcerans* to survival in host environments that are screened by immunological defence mechanisms.

Introduction

The emerging pathogen *Mycobacterium ulcerans* is the causative agent of Buruli ulcer, a mycobacterial disease of skin and soft tissue with the potential to leave sufferers scarred and disabled. While it is endemic in more than 30 countries (26), the major disease burden falls on children living in poor rural communities of West Africa. Buruli ulcer is prevalent in areas neighbouring rivers, slow-flowing waters, and swamps, but the exact mode of transmission has remained elusive. This is partly attributable to a clonal population structure and an associated lack of high-resolution genetic fingerprinting methods for microepidemiologic studies.

M. ulcerans seems to have recently evolved via lateral gene transfer and reductive evolution from the fish disease-causing environmental species *Mycobacterium marinum* (40, 43). Particularly, it has acquired the virulence plasmid, pMUM001, encoding the genes for the synthesis of the macrolide toxin, mycolactone. This toxin has cytopathic and immunomodulatory properties and plays a decisive role in producing an extracellular infection after an initial phase within macrophages (4, 41, 42, 47). In addition, *M. ulcerans* has undergone extensive gene loss due to DNA deletions, DNA rearrangements, and pseudogene formation, which apparently drives its evolution toward a niche-adapted specialist (27, 34, 39). Previous findings suggest that *M. ulcerans* lineages from different geographic areas reveal variations in virulence (27, 32; also F. Portaels, unpublished data).

The ESX-1 secretion system is required for the virulence of *Mycobacterium tuberculosis* and related pathogenic mycobacteria. It comprises the 6-kDa early secretory antigenic target protein (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), which are among the strongest T-cell response elicitors in tuberculosis patients (7, 8). The genes encoding these proteins are localized on the region of difference 1 (RD1) locus, which is intact in virulent members of the *M. tuberculosis* complex but absent from the attenuated vaccine strain *Mycobacterium bovis* BCG ($\Delta RD1^{BCG}$) (21, 29). Similarly, the vole bacillus, *Mycobacterium microti*, was found to have a natural deletion ($\Delta RD1^{microti}$) overlapping the deletion $\Delta RD1^{BCG}$ (6, 18). The so-called extended RD1 encompasses most of the genes that form the ESX-1 secretion apparatus (7, 16, 17) or are crucial for both ESAT-6/CFP-10 secretion and virulence (7, 17, 19, 31). This secretion apparatus enhances virulence in *M. tuberculosis* and *M. marinum* infection by secretion of effector proteins into the

cytosol of infected macrophages (37), prevention of phagolysosomal maturation (28, 45), and cytolytic activity (24). On the other hand, infected individuals develop strong T-cell responses against these proteins, which seem to be relevant for immune protection (8). The 16-kDa heat shock protein HspX, or α-crystallin-like protein Acr, a dominant protein expressed during static growth in *M. tuberculosis*, is required for mycobacterial persistence within the macrophage. HspX is yet another potent immune response elicitor and suitable for detecting *M. tuberculosis* infection (14, 15, 20, 25, 35, 49).

In mycobacterial disease control, highly antigenic proteins serve both as targets for diagnostic tests and as candidate proteins for vaccine development (1, 8, 30). While being present in the sequenced *M. marinum* strain M (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/m_marinum), genes encoding ESAT-6, CFP-10, and HspX are absent from the genome of the sequenced Ghanaian *M. ulcerans* strain Agy99 (http://genopole.pasteur.fr/Mulc/BuruList.html). However, earlier data showed that some *M. ulcerans* isolates and other related mycolactone-producing mycobacteria harbour at least segments of these genes (32, 48). Recently, we have identified two distinct genetic lineages of *M. ulcerans*, with representatives of the ancestral lineage being phylogenetically closer to its progenitor, *M. marinum*, than members of the *M. ulcerans* classical lineage (27). Here, we have analyzed a worldwide collection of *M. ulcerans* strains belonging to these two lineages for the presence of *esxA*, *esxB*, and *hspX* and their surrounding genomic regions.

Materials and Methods

Mycobacterial strains and genomic DNA extraction.

M. marinum strain M was used for interspecies comparison. A worldwide strain collection of *M. ulcerans* had been used earlier for investigation of genomic strain variations (34). Although several attempts to differentiate these strains achieved only low resolution (2, 3, 11, 22, 23, 38, 44), this collection of patient isolates was shown to be divided in two lineages displaying major genomic differences (27). In this study, we used *M. ulcerans* clinical isolates of both lineages as follows. For the classical lineage, the following strains were used: Ghana Agy99, Ghana ITM (Institute of Tropical Medicine, Antwerp, Belgium) 970321, Ghana ITM 970359, Ghana ITM 970483, Ivory Coast ITM 940662, Ivory Coast ITM 940815, Ivory Coast ITM 940511, Benin ITM 970111, Benin ITM 940886, Benin ITM 940512, Benin ITM 970104, Democratic Republic of Congo (DRC) ITM 5150, DRC ITM 5151, DRC ITM 5155, Togo ITM 970680, Angola ITM 960657, Angola ITM 960658, Papua New Guinea (PNG) ITM 941331, PNG ITM 9537, Malaysia ITM 941328, Australia ITM 941324, Australia ITM 941325, Australia ITM 941327, Australia ITM 9549, Australia ITM 9550, Australia ITM 8849, Australia ITM 940339, Australia ITM 5142, and Australia ITM 5147. For the ancestral lineage, the following strains were used: China ITM 980912, Japan ITM 8756, French Guiana ITM 7922, Surinam ITM 842, and Mexico ITM 5143. The presence of the specific PCR products obtained with primer pairs CH5/CH4 and CH3/CH4 (that exclude each other by design) (see Fig. 1) occurred concomitantly in strains DRC ITM 5151 and PNG ITM 941331. Since also variable-number tandem repeat typing analysis indicated that these strains are impure, we excluded these strains from further analysis.

Bacterial pellets of about 60 mg (wet weight) were heat inactivated for 1 h at 95°C in 500 µl of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 5% monosodium glutamate) and sequentially treated with 17 M lysozyme (for 2 h at 37°C) and 0.3 M proteinase K in proteinase K buffer (1 mM Tris-HCl, 5 mM EDTA, 0.05% sodium dodecyl sulfate, pH 7.8; overnight at 45°C). After digestion, the samples were subjected to bead-beater treatment (7 min at 3,000 rpm; Mikro-Dismembrator; B. Braun Biotech International, Melsungen, Germany) with 300 µl of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK). DNA was extracted from the supernatants by phenol-chloroform (Fluka, Buchs, Switzerland) extraction and

subjected to ethanol precipitation. DNA concentration was measured by determining the optical density at 260 nm (GeneQuant spectrophotometer; Pharmacia Biotech, Cambridge, United Kingdom).

DNA methods.

PCR was performed using FirePol 10× BD buffer and 0.5 μl of FirePol Taq polymerase (Solis BioDyne, Tartu, Estonia), 2.5 ng of genomic DNA or the equivalent volume of nuclease-free water as a negative control, a 0.6 μM concentration of each forward and reverse primer, 1.7 mM $MgCl_2$, and a 0.3 mM concentration of each deoxynucleoside triphosphate in a total volume of 30 μl. PCRs were run in a GeneAmp PCR system 9700 PCR machine. The thermal profile for PCR amplification of *M. ulcerans* genomic DNA included an initial denaturation step of 95 to 98°C for 5 min, followed by 32 cycles of 95°C for 20 s, annealing at 58 to 65°C for 20 s, and elongation at 72°C for 30 s up to 4 min. The PCRs were finalized by an extension step at 72°C for 10 min. For experiments with more than 30 samples, Hot Star *Taq* (Qiagen AG, Hombrechtikon, Switzerland) was used according to the manufacturer's protocol. In order to retrieve PCR products that were subsequently subjected to sequencing, iProof high-fidelity DNA polymerase (Bio-Rad Laboratories, Hercules, CA) was used. PCR products were analyzed on 1 to 2% agarose gels by gel electrophoresis using ethidium bromide staining and the AlphaImager illuminator and AlphaImager software (Alpha Innotech, San Leandro, CA). Primers as summarized in Table 1 were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR fragments produced for analysis of unknown genomic sequences were purified using a NucleoSpin purification kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and subjected to direct sequencing or cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA), transformed into JM109 (Sigma Aldrich, Buchs, Switzerland) bacterial cells, and sequenced after DNA preparation (Miniprep Kit; Sigma Aldrich, Buchs, Switzerland). Variable-number tandem repeat typing analysis undertaken for confirmation of strain identities was performed according to the method of Stragier et al. (44). Sequencing was performed using a BigDye kit and an ABI Prism 310 genetic sequence analyzer (Perkin-Elmer, Waltham, MA). All gene sequences were reproduced and subjected to alignment and comparison with an ABI Prism Autoassembler, version 1.4.0 (Perkin-Elmer, Waltham, MA).

Data analyses and bioinformatics.

Retrieved sequences were compared to the BuruList (http://genopole.pasteur.fr/Mulc /BuruList.html) and the *M. marinum* (http://www.sanger.ac.uk/cgi-bin/BLAST/ submitblast/m_marinum) BLAST servers and analyzed using the Sequence Manipulation Suite (http://bioinformatics.org/sms/index.html), the sequence alignment tool BLAST 2 sequences (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi), the multiple sequence alignment website Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html), and the Artemis software program, release 9 (The Wellcome Trust Sanger Institute, Hinxton, United Kingdom) (36). The sequences for *M. tuberculosis* were retrieved from the following Web page: http://www.sanger.ac.uk/Projects/M_tuberculosis. Linear genomic comparison was performed using the Artemis comparison tool software, release 6 (9).

Nucleotide sequence accession numbers.

The sequences of the indicated genes from *M*. *ulcerans* strains have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the following accession numbers (the associated protein is shown in parentheses): for *hspX* (HspX), accession numbers EU257156, EU257157, EU257158, EU257159, and EU257160; for *esxA* (ESAT-6), accession numbers EU257151, EU257152, EU257153, EU257154, and EU257155; and for *esxB* (CFP-10), accession numbers EU257146, EU257147, EU257148, EU257149, and EU257150. Accession numbers correspond to genes from the Japan 8756, China 980912, Surinam 842, French Guiana 9722, and Mexico 5143 strains, in respective order. Note that the annotated *hspX* gene in *M*. *ulcerans* Agy99 is an orthologue of *M. tuberculosis htpX* and that the *M. tuberculosis hspX* orthologue is not present in strain Agy99.

Results

Presence of *esxB-esxA* in *M. ulcerans* strains of the ancestral lineage.

Blast searches of the partially annotated genome of *M. marinum* M (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/m_marinum) showed that this strain contains two copies of the *esxB-esxA* (CFP-10-ESAT-6) gene cluster. Both copies are deleted from the genome of the African *M. ulcerans* isolate Agy99 (43). The corresponding two RDs between the genome sequences of the two mycobacterial species have been designated MURD152 (*M. marinum* genome position 6489253 to 6592034) and MURD4 (*M. marinum* genome position 218302 to 230285) (43).

Compared to *M. marinum* M, the *M. ulcerans* Agy99 genome has a 2.8-kb deletion in MURD152, which is associated with a large inversion at the 5′ end of the deletion (Fig. 1A). To test whether all *M. ulcerans* lineages share this genome constellation in MURD152, we screened a comprehensive *M. ulcerans* strain collection of worldwide origin by PCR analysis using a primer pair (CH3 and CH4) that yields a PCR product of 162 bp only when MURD152 is deleted and flanked by the inverted sequence (Fig. 1A and B). Whereas members of the ancestral lineage (strains from Asia, South America, and Mexico) were negative, members of the classical lineage (strains from Africa, Papua New Guinea, Malaysia, and Australia) were positive, except for strain Australia 9549, which has a larger deletion in this region (see below). Likewise, a PCR using a primer pair (CH8 and CH9) specific for the sequence constellation of strain Agy99 in MURD4 revealed a PCR product of 1,712 bp only for representatives of the classical and not for members of the ancestral lineage (Fig. 1B), demonstrating genomic diversity between the two *M. ulcerans* lineages in this locus.

A PCR with primers (CH1 and CH2) corresponding to the 5′ end of the *esxB* coding sequence and the 3′ end of the *esxA* coding sequence (Fig. 1A) yielded a PCR product of the expected size of 610 bp with genomic DNA from the *M. marinum* control and in all *M. ulcerans* strains belonging to the ancestral lineage (Fig. 2). Primers corresponding to the flanking regions of either the MURD4- or the MURD152-associated *esxB-esxA* gene cassette were used to determine the localization of this cluster in the genomes of these *M. ulcerans* strains (Fig. 2). Results indicated that *esxB-esxA* of the Asian and South American strains is located in MURD152, whereas in the Mexican strain the gene cluster is located in MURD4

(Fig. 2). These localizations were verified by PCR analyses extending several kilobases further into the flanking regions. While in the Asian and South American haplotypes the respective *M. marinum* MURD152 genome constellations were found, the cluster was flanked in the case of the Mexican haplotype by the MURD4-associated sequences of *M. marinum*.

Unique deletions in MURD152 in strains 5143 from Mexico and 9549 from Australia.

While MURD152 *esxB-esxA* is deleted from Mexican strain 5143 (Fig. 2), no PCR product specific for the MURD152 constellation of the strains belonging to the classical lineage was obtained with primers CH3 and CH4 (Fig. 1B), giving evidence for a larger deletion. A PCR analysis with primers corresponding to different positions of the genomic sequences flanking MURD152 demonstrated that the Mexico 5143 strain has a deletion (Fig. 3, ΔRD13A) that is replaced by an IS*2404* element. This insertion-deletion (indel) event can have occurred either from an *M. marinum* M-like genome constellation or from an *M. ulcerans* Agy99-like constellation (loss of 41.8 kb or of 8 kb, respectively). The DNA sequences flanking ΔRD13A in the Mexican strain have a slightly higher identity to the corresponding sequence stretches of *M. ulcerans* Agy99 than to those of *M. marinum* M (98% versus 94% over 986 bp).

Failure to obtain a PCR product with both the CH1/CH2 and the CH3/CH4 PCR primers for the Australian strain 9549 (Fig. 1) provided evidence for yet another deletion type within the MURD152 region. PCR analysis using primers located in the sequences flanking the corresponding region in the *M. ulcerans* Agy99 genome led to the characterization of a deletion of 13,662 bp including an IS*2404* element on each of the ends of the deleted DNA segment (Fig. 3, ΔRD13B). The deleted DNA stretch was, in strain Australia 9549, replaced by an IS*2404* element that, according to sequence analysis, differed from both versions of IS*2404* in Agy99 that were deleted in the ΔRD13B deletions.

Sequence variation in ESAT-6 and CFP-10.

PCR products obtained with primers corresponding to MURD locus-specific flanking regions and comprising the respective *esxB-esxA* clusters (Fig. 2) were sequenced. Deduced amino acid sequences of all versions of *M. ulcerans* ESAT-6 and CFP-10 encoded in MURD4 (Mexico 5143) or MURD152 (South American and Asian strains)

were compared with the *M. marinum* M sequences in the two loci (Fig. 4; see also the supplemental material). As expected, the translated ESAT-6 amino acid sequence of the Mexican strain clustered to and was identical with the MURD4-associated *M. marinum* M sequence (Fig. 4B). While the four MURD152-associated *M. ulcerans* ESAT-6 sequences of the Asian and the South American strains were identical to each other, their amino acid sequences differed at six positions from the MURD152-associated *M. marinum* sequence but only at two positions from the MURD4-associated *M. marinum* sequence (Fig. 4B). At the nucleotide level, the *esxA* genes of the Asian and South American strains appear as hybrids composed of an *M. marinum* MURD4 sequence stretch at the 5′ end and a MURD152 stretch at the 3′ end.

The two *M. marinum esxB* genes differed only at three nucleotide positions at the 5′ end (Fig. 4A), encoding CFP-10 proteins with identical deduced amino acid sequences (Fig. 4B). The *esxB* gene of the Mexican strain differed at four positions from the *M. marinum* M MURD4 locus but at only one position from the MURD152 locus. While the *esxB* gene sequences of the South American *M. ulcerans* strains were identical to the MURD152-associated sequence, a frameshift mutation has converted *esxB* of the Asian strains to a pseudogene (Fig. 4B).

Lack of the immunodominant HspX/Acr protein in the classical lineage of *M. ulcerans*.

Next, we screened the worldwide *M. ulcerans* strain collection for the presence of the coding sequence (CDS) encoding the immunogenic protein HspX (Acr) located in MURD92 (*M. marinum* genome position 4271366 to 4313737) (43). Using primers (CH14 and CH15) corresponding to the *hspX* flanking regions, a PCR product of 791 bp comprising the complete *hspX* gene was obtained for all members of the ancestral lineage but for none of the strains belonging to the classical lineage (not shown). Instead, amplification of a 469-bp PCR product (primers CH16 and CH17) obtained with a complementary PCR again demonstrated the presence of the Agy99 genome constellation (related to the MURD92 deletion) in all members of the classical lineage. While strains coming from the same geographical area had identical gene sequences, Asian and South American sequences differed slightly from each other and from the *M. marinum* sequence (Fig. 5A; see also the supplemental material). In

the case of the Mexican strain, nucleotide insertions resulted in a frameshift mutation leading to a truncated translation product (Fig. 5B).

Discussion

The *M. tuberculosis* proteins ESAT-6, CFP-10, and HspX are strong T- and B-cell immunogens. This makes them suitable targets for immunodiagnostic tests (7, 8, 14, 15, 20) and potentially also for subunit vaccine development (1, 30, 35). These approaches cannot be duplicated for Buruli ulcer, since these proteins are not expressed by classical-lineage *M. ulcerans* strains that are found in areas of endemicity in Africa and Australia and are responsible for the vast majority of clinical cases worldwide.

The genome of the *M. marinum* strain M harbours two *esxB-esxA* gene clusters at distant chromosomal locations, one in MURD4 and the other in MURD152. Such duplications are common for proteins of the Esx protein family (46). In this report we demonstrate that all analyzed *M. ulcerans* strains belonging to the ancestral lineage have lost only one copy of the *esxB-esxA* cassette: the Asian and South American strains have lost the MURD4 copy and the Mexican strain has lost the MURD152 copy. Furthermore, a frameshift mutation has converted the remaining *esxB* gene of the Asian strains to a pseudogene. The basis for the high degree of identity of the N-terminal *esxA* nucleotide sequence located in the MURD152 locus in the South American and Asian haplotypes with the *M. marinum* MURD4 sequence is unclear, but the finding implies a history of homologous recombination between the two copies of the *esxB* and *esxA* genes before loss of the MURD4 region. Members of the classical lineage have lost both copies, probably in a bottleneck situation that forged this lineage.

Since MURD152, MURD92, and MURD4 show genomic differences not only between *M. marinum* and *M. ulcerans* but also within *M. ulcerans* strains, we designated these RDs RD13, RD14, and RD15, respectively, in continuation of the previously assigned RDs within the species *M. ulcerans* (34). A detailed alignment of the chromosomal organization in RD13, which corresponds to RD1 in *M. tuberculosis*, is shown in Fig. 3. These RDs represent hot spots of genetic variation potentially suitable for performing genetic fingerprinting of *M. ulcerans*.

In addition to the previously identified five *M. ulcerans* indel haplotypes (27, 34), strain Australia 9549 was identified as representing a sixth indel haplotype, which is defined by ΔRD13B.

In MURD152 alone, at least three different deletion events are responsible for the indel diversity within *M. ulcerans* (Table 2). When this region was analyzed for variations among a collection of mycolactone-producing mycobacteria, an unclear situation was suggested for a Mexican strain (48). Here, we show that the deletion of 8 kb replaced by an IS*2404* element (ΔRD13A) in the Mexican strain (or 41.8 kb with respect to the *M. marinum* backbone) differs from the MURD152 deletion in Agy99. This deletion is independent of yet another extended deletion of 13.7 kb (ΔRD13B) in this genomic region in the Australia 9549 strain. The latter deletion is also replaced by an IS*2404* element and displays a second, large sequence polymorphism within Australian isolates in addition to the previously described RD3 (27, 34). It will be worth investigating the distribution of this indel polymorphism within a collection of Australian *M. ulcerans* isolates using the primer pair combination CH10/CH11, demonstrating the presence of the ΔRD13B deletion, and both CH10/CH12 and CH13/CH11, displaying positive results for strains with the sequence configuration of Agy99 (Fig. 3).

The described deletions also encompass CDSs surrounding the *esxA*, *esxB*, and *hspX* genes, indicating loss or modification of molecular apparatuses or pathways. First, PE35, essential for secretion (7), was lost in both MURD152 and ΔRD13A and is also commonly deleted in $\Delta RD1^{BCG}$ and $\Delta RD1^{microti}$ (Fig. 3). Second, many of the genes of the ESX-1 secretion system (the genes Rv3866/MMAR_5441 through Rv3881/MMAR_5457/*espB*, corresponding to the extended RD1 region) are equally affected by deletions ΔRD13A and/or MURD151 through MURD153, namely, the AAA protein family members Rv3868/MMAR_5443, Rv3871/MMAR_5446, and Rv3877/MMAR_5452 (7, 17, 19). Members of the classical lineage omit an MMAR_5457 orthologue in MURD153, which was recently described as a secreted product and renamed *espB* (31). Also in MURD92, *hspX* was jointly deleted with the co-regulated Rv2032/nitroreductase gene (33).

As for ESAT-6 and CFP-10, we also found for HspX different genetic mechanisms that have led to loss of expression, comprising both deletions of genomic sequences and single-base differences (Table 2). Many of the sequence variations across the *M. ulcerans* haplotypes that led to the loss (of function) of these highly immunogenic proteins appear to have emerged independently of each other. This may indicate a counterselection for expression of these proteins. HspX seems to be a negative growth regulator involved in hypoxic shift-down to promote the non-replicating

persistence of *M. tuberculosis* (15, 20, 25). Both ESAT-6 and CFP-10 were shown to be virulence factors of *M. tuberculosis*, and their loss reduces infectivity due to the dysfunction of the ESX-1 secretion apparatus (5, 10, 12, 13). The mycolactone-producing and largely extracellular *M. ulcerans* has a profoundly different survival strategy in mammalian hosts than the intracellular *M. tuberculosi*s; therefore, it is most likely that the pathogenicity of *M. ulcerans* for mammalian hosts is due to other virulence factors. Thus, our data suggest that functional disruption or complete loss of major targets of the immune response may confer a selective advantage to this emerging pathogen. Still, it is currently unclear whether pathogenicity for mammalian hosts, i.e., shedding into the environment from chronic wounds, contributes significantly to the survival of the species *M. ulcerans*. However, the observed loss of expression of highly immunogenic proteins caused by a variety of genomic changes may represent an indication that immune selection plays a role in the adaptation of *M. ulcerans* to a more stable environment.

Acknowledgements

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Tables

Table 1: Primers used in this study and description of respective PCR products

All primers are listed in 5'-3' orientation.

RD	Locus	Description of PCR product	Product size (bp)	Primer 1		Primer 2	
				Name	Sequence (5'–3')	Name	Sequence (5'–3')
13/14	MURD4/MURD152	Presence of *esxB-esxA* cluster in MURD4 and/or MURD152	610	CH1	TGAAGACCGATGCCGCTAC	CH2	AACATCCCCGTGACGTTG
13	MURD152	MURD152 deletion as in Agy99	162	CH3	CGTTGGGGTGAATTTCTTTG	CH4	AGTCTGACGGCGACTCATCT
13	MURD152	Presence of *esxB-esxA* cluster in MURD152	968	CH5	TTGGCGAGGAAAGAAAGAGA	CH4	AGTCTGACGGCGACTCATCT
14	MURD4	Presence of *esxB-esxA* cluster in MURD4	810	CH6	GACCCAAAGAGATAGAGAGTCCA	CH7	TCATCGGTGTCGGTGTAGTG
14	MURD4	MURD4 deletion as in Agy99	1,712	CH8	GACCCAGACGATGTGAATTG	CH9	GGAGCATGTTCACGATGTTG
13	MURD152	Deletion ΔRD13A	2,354	CH18	CAGTTATCGTGCGGGAATTT	CH19	ATCGGGAGAAAGACCGAAGT
13	MURD152	Deletion ΔRD13B	1,650	CH10	CTGGCGGAAACAACAACC	CH11	TCCTGGTCAAGTTGGAGACC
13	MURD152	MURD152 deletion as in Agy99	3,198	CH10	CTGGCGGAAACAACAACC	CH12	GCCGCTAACTTGAAGAATCG
13	MURD152	MURD152 deletion as in Agy99	1,662	CH13	TTCTCGCTCAATCTCCCCTA	CH11	TCCTGGTCAAGTTGGAGACC
15	MURD92	Presence of *hspX* in MURD92	791	CH14	GGCGCTTAAACCGGTCGTTG	CH15	CGCCAAACCCAGGACAATCA
15	MURD92	MURD92 deletion as in Agy99	469	CH16	AGCTGGCTAGCGTCGTACC	CH17	CCCAAAGCTCGTAGATCAGC

Table 2: Genomic deletions and amino acid changes in CDSs of immunogenic proteins

Strain, lineage, and haplotype	Characteristics of the indicated gene (protein) by RD and locus[a]				
	RD13 (MURD152)		RD14 (MURD4)		RD15 (MURD92), *hspX* (HspX)
	esxA (ESAT-6)	*esxB* (CFP-10)	*esxA-1* (ESAT-6)	*esxB-1* (CFP-10)	
M. marinum M	CDS	CDS	CDS	CDS	CDS
M. ulcerans ancestral lineage strains					
South America	CDS; A17S, Q19G, T23G, R52Q, N57K, S68A	CDS	Deletion[b]	Deletion[b]	CDS; A105S
Asia	CDS; A17S, Q19G, T23G, R52Q, N57K, S68A	Frameshift mutation (pseudogene)	<2.8-kb deletion[c]	<2.8-kb deletion[c]	CDS; V139F
Mexico	ΔRD13A	ΔRD13A	CDS[d]	CDS[d]	Frameshift mutation (pseudogene); D64A, L74R
M. ulcerans classical lineage strains					
Agy99, Africa, Australia	MURD152 deletion	MURD152 deletion	MURD4 deletion	MURD4 deletion	MURD92 deletion
Australia 9549	ΔRD13B	ΔRD13B	MURD4 deletion	MURD4 deletion	MURD92 deletion

Figures

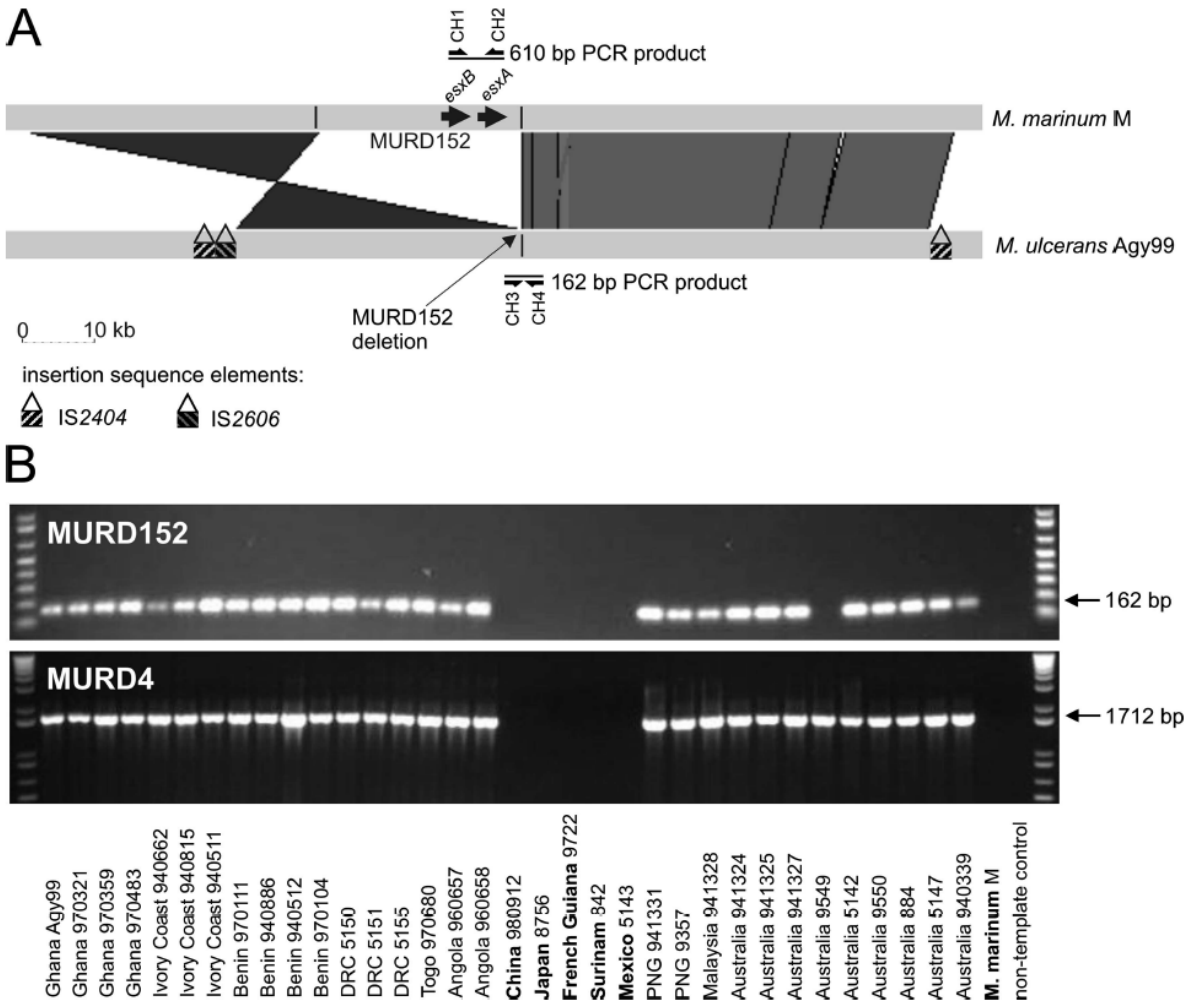

Figure 1: Confirmation of the MURD-specific deletions affecting *esxB* (CFP-10) and *esxA* (ESAT-6) in an *M. ulcerans* worldwide strain collection. (A) Schematic view of an alignment of *M. marinum* M and *M. ulcerans* Agy99 genomic sequences displayed by the Artemis comparison tool (9). Regions of conformity are shown in parallel gray plains, an inverted DNA segment is depicted as an inverted surface, and white areas represent unique sequences like MURD152, which is present only in *M. marinum* M and is deleted from *M. ulcerans* Agy99. Indicated are the genes *esxB* and *esxA* and the PCR primers (CH1 through CH4) used for this experiment. (B) PCR products of 162 bp or 1,712 bp proved the MURD152 deletion of 2.8 kb and the MURD4 deletion of 12 kb, respectively.

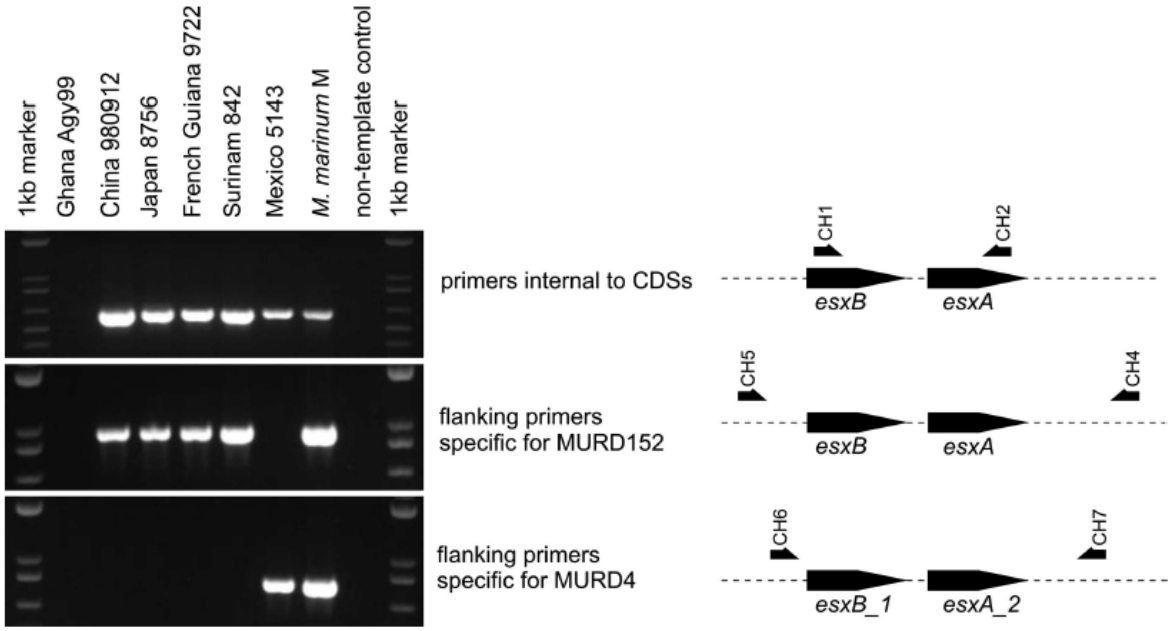

Figure 2: Localization of the two *esxB-esxA* clusters in the genomes of strains of the *M. ulcerans* ancestral lineage. Positions of the corresponding primers are indicated for the PCR product of the *esxB-esxA* cluster, where CH1 and CH2 correspond to sequences within the CDSs of both locations, and of the slightly larger PCR products amplified with flanking primers specific for either MURD152 or MURD4 (Table 1).

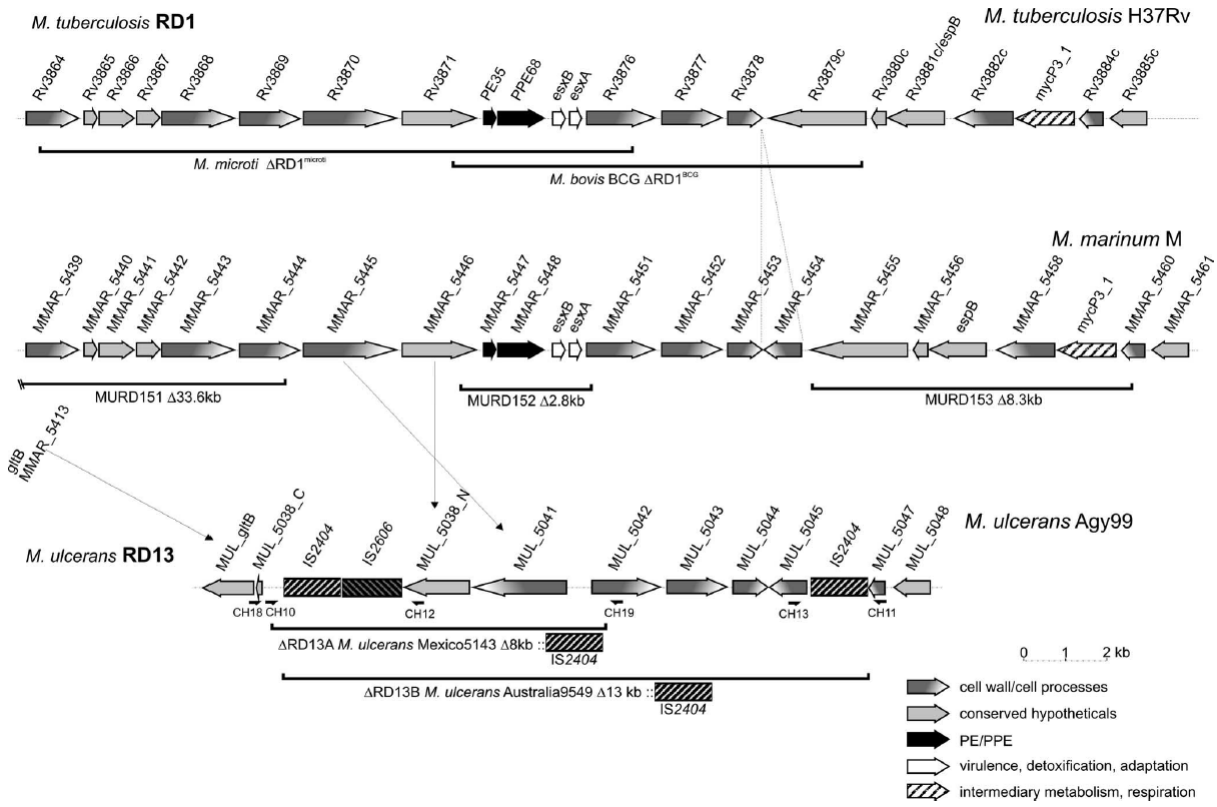

Figure 3: Chromosomal organization of CDSs in RD13 including deletional variations between *M. ulcerans* and other mycobacteria. Gene names are indicated for *M. tuberculosis* (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=224), *M. marinum* (http://www.sanger.ac.uk/Projects/M_marinum/), and *M. ulcerans* (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=ShowDetailView&TermToSearch=16230), and orthologous genes are aligned. RD13 of *M. ulcerans* corresponds to RD1 in *M. tuberculosis*. Deletions in *M. bovis* BCG, *M. microti*, and various *M. ulcerans* strains are indicated by solid bars as marked

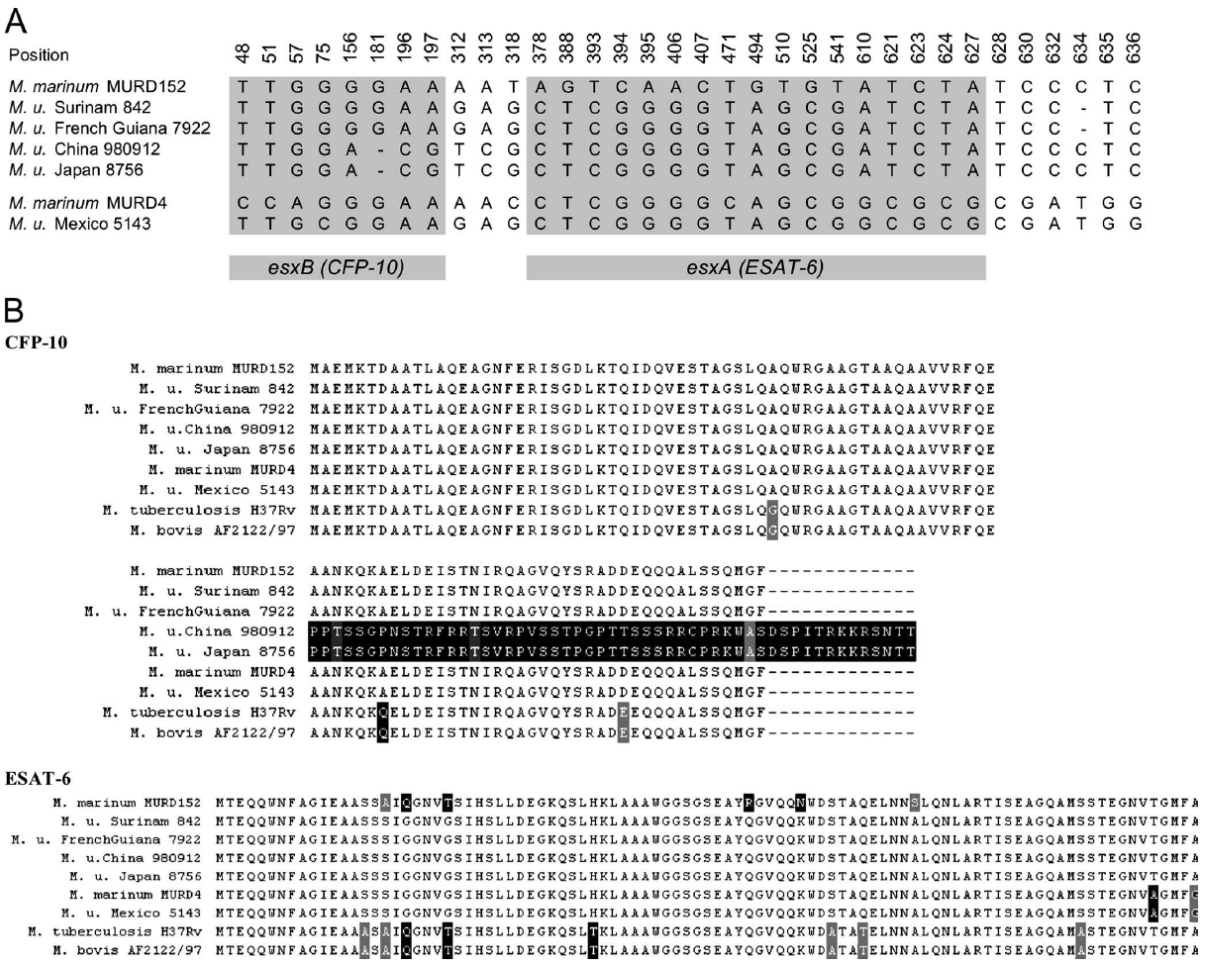

Figure 4: Nucleotide variations (A) and amino acid sequence alignments (B) in *esxB* and *esxA* CDSs and their gene products (CFP-10 and ESAT-6, respectively).

Position 1 of the nucleotide alignment reflects the start of the gene *esxB*. For the DNA sequences, only differing nucleotides are shown (positions as indicated). For whole-sequence alignments, see the supplemental material. Orthologous sequences of *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 are included in the amino acid alignments.

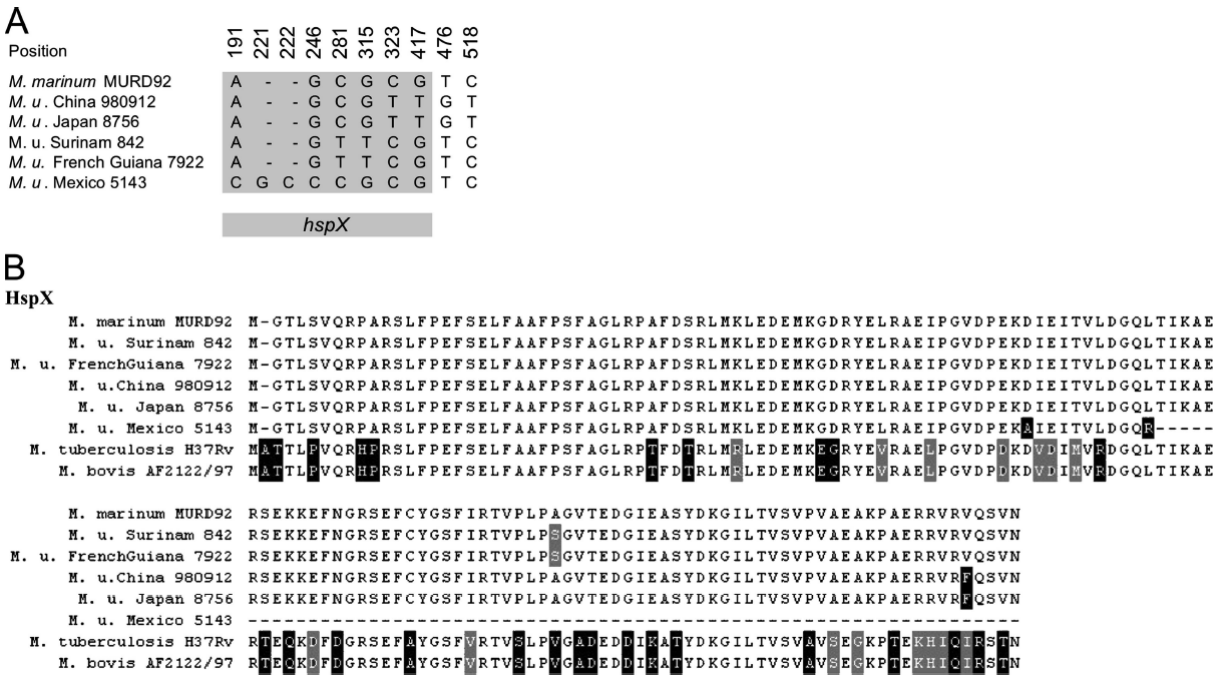

Figure 5: Nucleotide variations (A) and amino acid sequence alignments (B) in the *hspX* CDS and its gene product. Position 1 of the nucleotide alignment reflects the start of the gene. For the DNA sequences, only differing nucleotides are shown (positions as indicated). For whole-sequence alignments see the supplemental material. Orthologous sequences of *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 are included in the amino acid alignments.

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Chapter 8

Optimized Method for Preparation of DNA from Pathogenic and Environmental Mycobacteria

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Abstract

Genomic studies on pathogenic and environmental mycobacteria are of growing interest for understanding of their evolution, distribution, adaptation, and host-pathogen interaction. Since most mycobacteria are slow growers, material from in vitro cultures is usually scarce. The robust mycobacterial cell wall hinders both experimental cell lysis and efficient DNA extraction. Here, we compare elements of several DNA preparation protocols and describe a method that is economical and practical and reliably yields large amounts—usually 10-fold increased compared to earlier protocols—of highly pure genomic DNA for sophisticated downstream applications. This method was optimized for cultures of a variety of pathogenic and environmental mycobacterial species and proven to be suitable for direct mycobacterial DNA extraction from infected insect specimens.

Introduction

Mycobacterial diseases are a major health concern for humans (i.e., *Mycobacterium tuberculosis*, *M*. *leprae*, *M*. *ulcerans*, *M*. *avium*, and *M*. *paratuberculosis*) (4, 13, 18, 29, 30), livestock (*M*. *bovis* and *M*. *avium* subsp. *paratuberculosis*) (1, 3), fisheries (*M*. *marinum*) (25), and wildlife (*M*. *avium*, *M*. *pinnipedii*, *M*. *microti*, *M*. *caprae*, and other species) (13, 20). Efficient methods for DNA preparation are required both for the identification and genotyping of such pathogens and for population genomics, which is developing into an important tool to study bacterial evolution, virulence, and epidemiology.

Extraction of mycobacterial genomic DNA is especially demanding since (i) many mycobacterial species are among the most extreme slow growers, accounting for small amounts of starting material, and (ii) a robust and waxy cell wall renders mycobacteria difficult to lyse. Published protocols for mycobacterial DNA preparations and commercially available extraction kits are mainly designed for the isolation of small amounts of genomic material suitable for conventional PCR application (2, 7, 9, 11, 14, 15, 23, 24, 27, 28, 33), such as for testing of potentially contaminated milk (6, 8, 17). However, such DNA quantities and qualities are usually not sufficient for more sophisticated molecular analyses.

M. *ulcerans*, the causative agent of the devastating human skin disease Buruli ulcer, is one of the slowest growers among mycobacterial species, and the development of molecular tools is crucial for studying its transmission and microepidemiology. The objective of this study was to develop an optimized extraction protocol for DNA of both high quantity and quality from scarce material of in vitro-cultivated *M*. *ulcerans* disease isolates. We compared elements of several protocols and developed a DNA preparation method that is optimized in each individual step and thus ready to use for virtually all mycobacterial species to yield a maximum of pure genetic material. In addition, we applied the established method to cultures of a variety of pathogenic and environmental mycobacterial species and tested it by isolating DNA from insects experimentally infected with *M*. *ulcerans*.

Materials and Methods

Mycobacterial strains and preparation of mycobacterial cell suspensions.

The strains used for this investigation and their origins are as follows: *M*. *ulcerans* Agy99, Malaysia 1615, and Japan 753 (21); Ghana IFIK1066089, Ghana Nm50/04, Ghana Nm51/04, Ghana Nm53/04, Ghana Nm74/02, Ghana Nm97/02, Ghana Nm98/02, Ghana Nm103/02, and Mexico IFIK 973880 (this study); Ghana Nm18/02, Ghana Nm21/02, Ghana Nm31/04, Ghana Nm38/02, and Ghana Nm59/02 (10); and Japan ITM 8756, French Guiana ITM 7922, and Surinam ITM 842 (22); *M*. *marinum* M, ATCC 927, CC240299, and DL240490 and *M*. *pseudoshottsii* L15 (21); *M*. *liflandii* XT128 (32); *M*. *tuberculosis* Pasteur 14001.0001 (5); and *M*. *bovis* BCG ATCC35734 (5). Mycobacteria were obtained from cultures as described earlier (19, 31), resuspended in phosphate-buffered saline (PBS; pH 7.4), and heat inactivated at 95°C for 60 min. Note that pathogenic mycobacteria need to be processed under appropriate biosafety containment. To avoid cross-contamination, 1.5-ml screw-cap tubes were used. Samples were centrifuged for 5 min at 2,500 × *g* to remove residual PBS.

Extraction of mycobacterial DNA from pellets.

Mycobacterial pellets were resuspended in 300 µl of lysis buffer A, B, or C (buffer A contained 5% monosodium glutamate, 50 mM Tris [pH 8.5], and 25 mM EDTA; buffer B contained 15% sucrose, 50 mM Tris [pH 8.5], and 50 mM EDTA; and buffer C contained 4 M guanidine isothiocyanate [GITC] and 50 mM Tris [pH 7.2]) and then incubated with different amounts of lysozyme. After incubation at 37°C for 1 h, sodium dodecyl sulfate (SDS) and proteinase K (PK) were added at different end concentrations and the mixture was further incubated at 37°C for 1 h, followed by enzyme inactivation at 70°C for 5 min. Some pellets were preincubated with chloroform-methanol (MeOH) at a 2:1 ratio for delipidation. Various matrix materials (200 µl of 0.1-mm zirconia beads [BioSpec Products, Bartlesville, OK] or 0.5-mm or 1.4-mm ceramic or glass beads [Bertin Technologies, Montigny-le-Bretonneux, France]) were used, and samples were homogenized with a mechanical bead beater device, the Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany) for 2 to 7 min at 2,000 to 3,000 rpm or the Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) at conditions ranging from 2 × 40 s

at 5,000 rpm to 3 × 30 s at 6,800 rpm. Supernatants were transferred to new 1.5-ml reaction tubes and subjected to phenol-chloroform (Fluka, Buchs, Switzerland) extraction and chloroform purification (Fluka, Buchs, Switzerland). For this, addition of 500 µl of phenol-chloroform or chloroform was followed by a vortexing step and centrifugation at room temperature for 5 min at 4,000 rpm. After isopropanol or ethanol (EtOH) precipitation at −70°C for >30 min, DNA pellets were resuspended in 100 µl nuclease-free water. Alternatively, mycobacterial purification kits (Promega Wizard [Promega AG, Dübendorf, Switzerland], Sigma GeneElute [Sigma-Aldrich, Buchs, Switzerland], and the BD GeneOhm lysis kit [Becton Dickinson Biosciences, Allschwil, Switzerland]) were used according to the manufacturers' protocols, without or in combination with mechanical treatment.

For further purification and quality control, extracted DNA was freed from residual RNA by incubation with 1.5 mg/ml RNase A (Fermentas, St. Leon-Rot, Germany) for 2 min at 37°C and purified from degradation products, residual solvent, and protein contaminants with the QIAmp DNA purification MiniKit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturers' protocols. For genome sequencing applications, DNA was concentrated with a Concentrator 5301 (Vaudaux-Eppendorf AG, Basel, Switzerland).

Experimental infection of aquatic insects and DNA extraction.

Wild-caught insects belonging to the family *Naucoridae* (*Naucoris cimicoides*) were collected from swamps in western France. They were housed in an aquarium—filled with water of their natural environment—at 28°C and a photoperiod of 12 h each light and dark without any feeding for 7 days. These insects were then fed with a 15- to 20-day-old grub of *Phormia terrae novae* (Verminière de l'Ouest, Tremblay, France) that was infected by inoculation with 10^6 CFU of *M*. *ulcerans* in a volume of 30 µl with a 25-gauge needle. Alternatively, *Naucoris* insects were directly inoculated in the coelomic cavity with 10^6 CFU of *M*. *ulcerans* in a volume of 30 µl with a 25-gauge needle (16). Insects were sacrificed with 70% cold EtOH, transferred to a 1.5-ml screw-cap tube, and processed for DNA extraction as described for mycobacterial pellets.

DNA quantification, amplification, and gel electrophoresis.

DNA concentration was determined with the NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE) by measuring the absorption at 260 nm, and the decontamination of DNA from solvents and proteins was estimated by measuring the absorption at 230 and 280 nm, respectively. Purity and fragmentation of the extracted genomic DNA were assessed by 1% agarose gel electrophoresis. Detection limits of purified genomic materials were assayed by PCR with primers targeting unique regions in the mycobacterial genomes (primers MK810 [TCTGTCAAGACAAGCCGATG], MK811 [GACTCGTGGTGATCGAGGAT], MK60 [ATCGTTTAGCGCATCGATCT], MK61 [CACAGGTCGACCCCAACTAC], MK63 [GTCGATGATCGCCTGTGGT], and MK35 [GTCGGCATCTTGTTGCTCA]). The presence of *M*. *ulcerans* in environmental insect specimens was tested with primers MU5 and MU6 (26) for detection of IS*2404* and MK289 (GTCGTAGATGTGGGCGAAA) and MK263 (GGTGCGGTTCCATTGAGA) for detection of IS*2606*. Primers were designed with the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed with FirePol 10× buffer and 0.5 μl FirePolTaq polymerase (Solis BioDyne, Tartu, Estonia), 10 ng genomic DNA, 0.6 μM each forward and reverse primer, 1.5 mM $MgCl_2$, and 0.4 mM each deoxynucleoside triphosphate in a total volume of 30 μl. PCRs were run in a GeneAmp PCR System 9700 PCR machine (Perkin-Elmer, Schwerzenbach, Switzerland). The thermal profile for PCR amplification of *M*. *ulcerans* genomic DNA included an initial denaturation step of 95°C for 5 min, followed by 32 cycles of 95°C for 20 s, annealing at 58°C for 30 s, and elongation at 72°C for 45 s up to 2 min 20 s. The PCR was finalized by an extension step at 72°C for 10 min, followed by the analysis of the PCR products on 1% agarose gels by gel electrophoresis with ethidium bromide staining and the AlphaImager illuminator (Alpha Innotech, San Leandro, CA).

Results and Discussion

Our first aim was to reduce the DNA extraction volume to 1.5-ml centrifugation tubes to facilitate rapid processing of large numbers of samples, in comparison to the commonly used volumes of 10 to 50 ml (2, 24). Extraction of DNA from samples with a wet weight of up to 20 mg in a volume of 1.5 ml turned out to be highly economical with respect to time and reagent consumption, while the use of more starting material did not result in a proportional increase in the amount of extracted DNA (Table 1).

Next, we adopted and evaluated elements of several protocols of mycobacterial DNA extraction (2, 9, 15, 23, 24) for a comprehensive method optimization (Table 1). It was reported earlier that removal of lipids and lipid-like surface proteins by chloroform-MeOH treatment preceding cell lysis improved DNA purity, especially of large-size DNA (2, 9, 12). We confirmed this but also experienced substantial loss of genomic material. In contrast to an earlier study (15), the type of lysis buffer used had no marked influence on the DNA yield. Likewise, changes in lysozyme and PK concentrations made no significant differences. However, we found that an increase in the concentration of SDS resulted in increasing DNA yields. A commercially available kit designed to chemically lyse mycobacteria for PCR purposes yielded only small amounts of DNA suitable for PCR. In contrast, high-velocity mechanical treatment turned out to be very efficient in cell wall disruption, as already reported in several studies (2, 17, 33). When comparing matrix materials for bead beater treatment, good results were obtained with both zirconia and glass beads and cell solubilization was best when using beads of small diameter. The DNA yields tended to be higher when using harsh conditions, i.e., highest speed conditions and homogenization times of the respective device (Table 1) (17). However, since high-velocity treatment jeopardizes shearing of DNA (2, 33), we recommend the performance of quality control by agarose gel electrophoresis after purification. Figure 1 shows that a combination of mechanical disruption and chemical solubilization of the waxy lipid and mycolic acid-containing cell wall renders the mycobacteria accessible for enzymatic lysis. Incubation with 4% SDS followed by mechanical disruption, a combination that was (to our knowledge) not used in any previous protocol, was here found to be essential for a high DNA yield (sample D). All protocols lacking one of these two treatments yielded strikingly smaller amounts of

DNA (samples B and C, respectively). When both steps were omitted, no DNA was extracted at all (sample A).

For purification, the properties of DNA binding to silica in the presence of chaotropic salts are generally used to circumvent the use of phenol-chloroform. However, silica-based commercial purification kits reached neither our elevated quantity nor quality requirements, even when applied after mechanical solubilization (Table 1; Fig. 1, samples G and H). Thus, although column use facilitates handling, we decided to use conventional phenol-chloroform extraction. Two steps of phenol-chloroform extraction were found to be crucial for removing protein and lipid contaminations from the genomic DNA, and an additional purification step with only chloroform helped in removing residual phenol (Table 1). A second round of this three-step phenol-chloroform extraction after EtOH precipitation led to enhanced DNA purity but reduced the overall yield (Fig. 1, sample E). Although the highest DNA yield and purity were represented by sample F (Fig. 1), where DNA was precipitated with isopropanol instead of EtOH, this protocol resulted, in most cases, in a yellowish, slimy pellet that was difficult to resuspend and thus prone to material loss during handling. In conclusion, when a maximum of extracted genetic material is required, the protocol described for sample D should be applied, which had a 260/280-nm ratio (~1.6) sufficient for most downstream applications. When a higher purity of DNA is required, a protocol including chloroform-MeOH pretreatment and/or a second round of phenol-chloroform extraction and EtOH precipitation is indicated, although it is associated with lower yields.

From these results, we derived an improved standard protocol (Table 2) that involves (i) the use of 20 mg (wet weight) of pellets; (ii) pretreatment with 10 mg/ml lysozyme, 200 µg/ml PK, and 4% SDS; (iii) mechanical disruption with 0.1-mm zirconia beads; (iv) two phenol-chloroform extractions, followed by chloroform purification; and (v) EtOH precipitation with two EtOH washing steps, followed by resuspension of the DNA pellet in 100 µl of nuclease-free water.

We applied the established DNA extraction method to cell pellets of a panel of *M. ulcerans* strains and other mycobacterial species including *M. marinum*, *M. pseudoshottsii*, *M. liflandii*, *M. tuberculosis*, and *M. bovis* BCG (Table 3). A mean yield of 713 ng genomic DNA per mg (wet weight) cells with a 260/280-nm ratio of 1.58 (standard deviation, 0.09) was obtained. This represents a 10- to 20-fold increase in DNA yield in our experiments compared to previous protocols, elements

of which we combined for optimization. We performed detailed quality control for DNA extractions from two strains, *M*. *ulcerans* Agy99 and Japan ITM 8756, representing the two distinct lineages of *M*. *ulcerans* (Fig. 2). Genomic DNA yields from 20-mg (wet weight) cell pellets were sufficient in quantity (typically, >5 µg; Fig. 2) and quality for whole-genome microarray hybridization and whole-genome sequencing analyses. Single-copy gene sequences of >2 kb could be easily amplified by PCR with 10 ng as the template (Fig. 2). Subsequent RNase treatment and genomic DNA column purification decontaminated the samples from RNA and small DNA fragments resulting from shearing of genomic DNA (Fig. 2).

In addition, we applied the optimized DNA extraction method to aquatic insects experimentally infected with *M*. *ulcerans*. Genomic DNA was sufficient in quantity and purity to specifically detect *M*. *ulcerans* in infected insects (Table 4), showing that the established method is suitable for direct processing of environmental samples.

In conclusion, we envision this protocol to facilitate the investigation of pathogenic and nonpathogenic mycobacteria sampled from both infected tissue and the environment. In combining and optimizing crucial elements of established DNA extraction methods, our ready-to-use protocol meets the challenging characteristics of slow growth and distinct cell wall composition of mycobacteria and greatly enhances both the yield and the purity of mycobacterial DNA preparations.

Acknowledgements

We are grateful to Pamela C. Small for provision of strains (*M*. *marinum* CC240299 and DL240490, *M*. *pseudoshottsii* L15, and *M*. *liflandii* XT128), Thomas Bodmer for cultivation of strain *M*. *ulcerans* IFIK1066089, and Dorothy Yeboah-Manu for *M*. *ulcerans* strains from Ghana.

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Tables

Table 1: Comparative advantages and/or disadvantages of various elements of DNA extraction protocols[a]

Protocol step and option(s)	Advantage(s) and/or disadvantage(s)
Reaction vol	
1.5, 2, or 10 ml	Minimized vol reduces time and reagent consumption, round bottom of 2-ml tubes complicates separation of EtOH precipitate from supernatant
Starting material	
5 to 40 mg (wet wt) pellets	Maximum DNA yield with 20 mg, no increase with more material in 1.5-ml reaction mixture vol
Delipidation pretreatment	
Chloroform-MeOH treatment preceding lysis	Enhanced purity but substantial loss of material
Lysis buffer	
A: 5% monosodium glutamate, 50 mM Tris (pH 8.5), 25 mM EDTA	GITC lysis buffer less efficient; otherwise, no differences encountered
B: 15% sucrose, 50 mM (Tris pH 8.5), 50 mM EDTA	
C: 4 M GITC, 50 mM Tris (pH 7.2)	
Disruption/digestion	
1 vs 10 mg/ml lysozyme, 100 vs 200 μg/ml PK	No differences encountered
SDS concn, 0.05 to 4%	Highest concn yields best result
Mechanical vs chemical lysis (BD GeneOhm kit)	Higher yields with mechanical lysis
High-velocity mechanical treatment	Yields efficiently increased
Mikro-Dismembrator S vs Precellys 24 homogenization device	Slightly enhanced yields with Precellys 24
Glass, zirconia, or ceramic beads	Higher yields with glass and zirconia beads
0.1-mm vs 0.5-mm beads	Higher yields with 0.1-mm beads
Homogenization conditions: 2–7 min at 2,000–3,000 rpm (Mikro-Dismembrator S), 2 × 40 s at 5,000 rpm to 3 × 30 s at 6,800 rpm (Precellys 24)	Harshest conditions result in highest yields
Purification	
Phenol-chloroform extraction	Purity efficiently increased
2 phenol-chloroform extraction steps followed by 1 chloroform purification step only	Purity further increased
Phenol-chloroform vs column purification	Higher yields and purity with phenol-chloroform but less labor intensive when using columns
Isopropanol vs EtOH precipitation	Higher yields with isopropanol but better to handle pellet with EtOH
Second round of phenol-chloroform extraction and EtOH precipitation	Higher DNA quality but substantial loss of DNA material

[a] These comparisons were made with *M. ulcerans* strain IFIK1066089. For details of the methodology, see Materials and Methods.

Table 2: Optimized protocol for preparation of mycobacterial DNA

Process and step	Vol (µl)
Cell wall disruption and digestion	
Transfer 20-mg (wet wt) pellet or environmental sample into a 1.5-ml screw-cap tube and wash with PBS if needed	~10
Resuspend in lysis buffer (15% sucrose, 0.05 M Tris [pH 8.5], 0.05 M EDTA)	300
Add lysozyme (stock, 100 mg/ml; end concn, 10 mg/ml):	50
Incubate for 1 h at 37°C	
Add SDS (stock, 20%; end concn, 4%)	100
Add PK (stock, 2.5 mg/ml; end concn, 0.2 mg/ml)	40
Total	500
Incubate for 1 h at 37°C	
Incubate for 5 min at 70°C	
Add matrix material (vol of ~150 µl glass or zirconia beads of 0.1-mm diam) and homogenize with mechanical treatment device	
Centrifuge at 14,000 rpm for 2 min	
Transfer supernatant to 1.5-ml reaction tube	
Phenol-chloroform extraction and EtOH precipitation	
Add phenol-chloroform-isoamyl alcohol (Tris saturated)	500
Vortex, centrifuge at 14,000 rpm for 5 min	
Collect supernatant	
Repeat phenol-chloroform extraction step	
Add chloroform-isoamyl alcohol	500
Vortex, centrifuge at 14,000 rpm for 5 min	
Collect supernatant	~200
Add 3 M sodium acetate	20
Add 100% EtOH	500
Freeze for >30 min at −70°C, centrifuge at 14,000 rpm for 30 min at 4°C	
Wash with 70% EtOH	700
Centrifuge at 14,000 rpm for 10 min at 4°C	
Repeat EtOH wash step	
Dry at 50°C, resuspend in nuclease-free H_2O	100
Quality control	
Measure DNA concn and purity with a spectrophotometer	
DNA concn: >100 ng/µl; protein: 260/280 nm, >1.8; detergent: 260/230 nm, >1.8	
Monitor quality on 1% agarose gel	

Table 3: DNA yields from a variety of mycobacterial species[a]

Species and lineage/ ecotype/strain	No. of isolates	Mean DNA yield (µg) (SD)	Mean DNA quality (260/280 nm ratio) (SD)
M. ulcerans classical lineage	34	19.83 (9.63)	1.63 (0.25)
M. ulcerans ancestral lineage	7	12.14 (7.72)	1.74 (0.24)
M. marinum human isolates	3	18.38 (6.58)	1.73 (0.03)
M. marinum fish isolates	4	19.99 (19.09)	1.40 (0.09)
M. pseudoshottsii L15	4	19.36 (11.44)	1.63 (0.14)
M. liflandii XT128	3	7.26 (5.86)	1.49 (0.21)
M. tuberculosis Pasteur 14001.0001	1	6.22 (NA)[b]	1.30 (NA)
M. bovis BCG ATCC 35734	1	10.78 (NA)	1.70 (NA)
Overall	57	14.25 (4.88)	1.58 (0.09)

[a] The optimized protocol was applied to 20 mg (wet weight) of pellets.
[b] NA, not applicable.

Table 4: Detection of *M. ulcerans* genomic DNA after extraction from infected aquatic insects[a]

TABLE 4. Detection of *M. ulcerans* genomic DNA after extraction from infected aquatic insects[a]

N. cimicoides sample	Total/no. (%) PCR positive	
	IS*2404*	IS*2606*
Uninfected control	0/10 (0)	0/10 (0)
Inoculated	8/8 (100)	8/8 (100)
Fed	4/7 (57)	4/7 (57)

[a] *N. cimicoides* insects were subjected to DNA extraction after experimental inoculation or feeding with *M. ulcerans* bacteria along with control insects (non-infected or sampled from the natural environment). *M. ulcerans* was identified by using two different specific loci.

Figures

SDS treatment[1]	×	×	yes	yes	yes	yes	yes	yes
Mechanical disruption[2]	×	yes	×	yes	yes	yes	yes	yes
Precipitation/purification	EtOH	EtOH	EtOH	EtOH	EtOH	isopropanol	column[4]	column[5]
Repeated Phe-Chl extraction[3]	×	×	×	×	yes	×	×	×

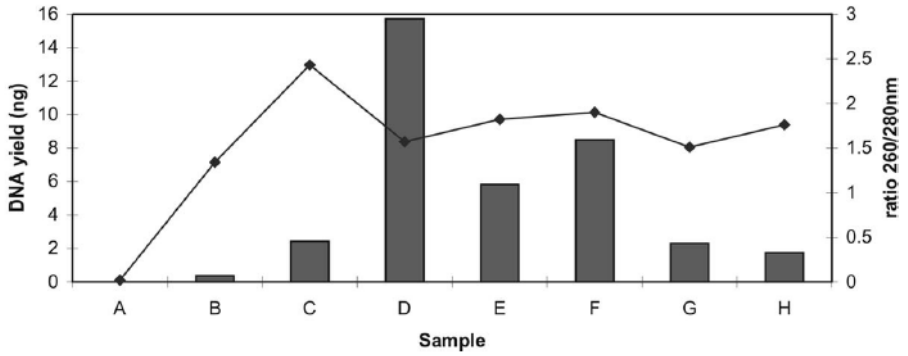

Figure 1: DNA yields (bars) and purity (line) achieved when testing different combinations of DNA extraction, purification, and precipitation procedures. Twenty-milligram (wet weight) pellets of *M*. *ulcerans* strain IFIK1066089 were used. (Superscript 1) SDS was applied to a final concentration of 4%. (Superscript 2) Mechanical disruption was performed with a Precellys 24 homogenizer at 6,800 rpm for 3 × 30 s with glass beads. (Superscript 3) Second round, after EtOH precipitation, of three-step phenol-chloroform (Phe-Chl) extraction. (Superscript 4) Promega Wizard. (Superscript 5) Sigma GeneElute. For details, see Materials and Methods.

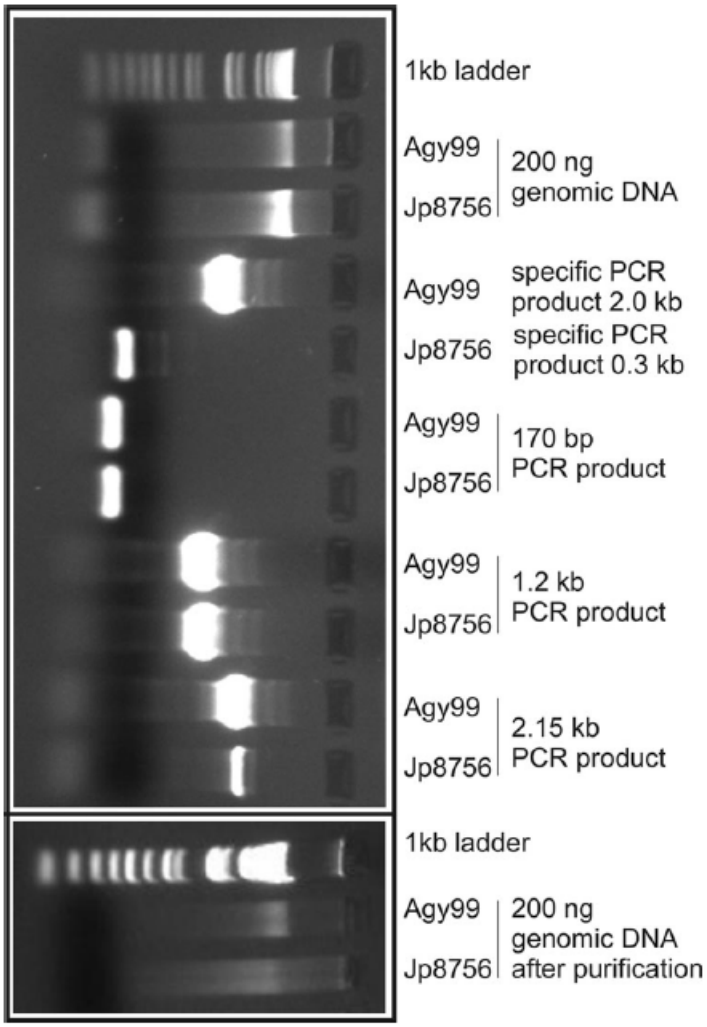

Figure 2: Quality control of DNA preparations from two *M*. *ulcerans* strains (Agy99 and Japan ITM 8756 [Jp8756]) by agarose gel electrophoresis. Shown are ethidium bromide stainings of isolated genomic DNA, four PCR products thereof, and RNase-treated and column-purified aliquots (right panel). Primer pair MK810/MK811 was designed to yield PCR product sizes specific for either Agy99 (2 kb) or Jp8756 (0.3 kb). Primer pairs MK60/MK61, MK60/MK63, and MK60/MK35 were designed to yield identical PCR products in both strains with increasing sizes (0.17, 1.2, and 2.15 kb, respectively).

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Chapter 9

Single nucleotide polymorphism typing of *Mycobacterium ulcerans* reveals focal transmission of Buruli ulcer in a highly endemic region of Ghana

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Abstract

Buruli ulcer (BU) is an emerging necrotizing disease of the skin and subcutaneous tissue caused by *Mycobacterium ulcerans*. While proximity to stagnant or slow flowing water bodies is a risk factor for acquiring BU, the epidemiology and mode of *M. ulcerans* transmission is poorly understood. Here we have used high-throughput DNA sequencing and comparisons of the genomes of seven *M. ulcerans* isolates that appeared monomorphic by existing typing methods. We identified a limited number of single nucleotide polymorphisms (SNPs) and developed a real-time PCR SNP typing method based on these differences. We then investigated clinical isolates of *M. ulcerans* on which we had detailed information concerning patient location and time of diagnosis. Within the Densu river basin of Ghana we observed dominance of one clonal complex and local clustering of some of the variants belonging to this complex. These results reveal focal transmission and demonstrate that micro-epidemiological analysis by SNP typing has great potential to help us understand how *M. ulcerans* is transmitted.

Author Summary

Mycobacterium ulcerans causes a destructive skin disease known as Buruli ulcer (BU), which has been reported from more than 30 tropical or subtropical countries, with the highest prevalence in Western Africa. Due to the striking genetic monomorphism of African *M. ulcerans* populations, conventional genetic fingerprinting methods have largely failed to differentiate isolates coming from the same BU endemic area.

Here we report a highly discriminatory fingerprinting method for *M. ulcerans* using a single nucleotide polymorphism-based genetic fine-typing technique. This method has enabled us for the first time to identify different *M. ulcerans* haplotypes within a BU endemic area. Linking the origins of *M. ulcerans* strains with the patients` residences unveiled the clustering of unique *M. ulcerans* haplotypes within the Densu river basin of Ghana. Results show, that haplotypes do not spread within a short time over the entire BU endemic region, but rather form independent focal transmission clusters.

Introduction

Infection with *Mycobacterium ulcerans* causes a chronic, necrotizing disease of the skin and the subcutaneous adipose tissue commonly known as Buruli ulcer [1]. This serious infectious disease remains a major health problem in many parts of the world, but in particular, in Western and Central Africa [2]. In spite of considerable research efforts made during the past few years transmission and environmental reservoirs of *M. ulcerans* are still incompletely characterized [1]. Endemic foci are usually linked to wetlands and riverine areas, which has lead to the assumption that *M. ulcerans* is an environmental mycobacterium and that micro-traumata of the skin may initiate infection [3]. However, isolation of the slow growing *M. ulcerans* from an environmental source has been achieved only once so far, from an aquatic insect [4]. PCR screening of environmental samples for the presence of *IS2404* has implicated insects such as biting aquatic hemiptera and mosquitoes in the transmission of *M. ulcerans* [5-7], but their positivity for *M. ulcerans* DNA in polymerase chain reaction (PCR) tests may be only an indicator for the presence of *M. ulcerans* or other genetically closely related mycobacteria in the environment. Although BU is known to develop in all age groups with a nearly equal gender distribution, children 15 years of age or younger make up at least 50% of all cases in Africa [8]. Occasional clustering of cases within families may reflect a common source of infection or increased genetic susceptibility to infection rather than human-to-human transmission. Seroepidemiological studies have indicated that infection with *M. ulcerans* may lead to disease only in a minority of exposed individuals [9].

Many genetic fingerprinting methods have been applied for *M. ulcerans*, including *IS2404*, *IS2606* and *IS2426* PCR [10,11], amplified fragment length polymorphism analysis (AFLP) [12], *IS2404* restriction fragment length polymorphism analysis (RFLP) [13,14], multi-locus sequence typing (MLST) [15-17], variable-number tandem repeat analysis (VNTR) [18-21], *IS2404-Mtb2* PCR [22], and large sequence polymorphisms [23]. Among these, AFLP [12] and VNTR typing [19,21] were the only methods to reveal any genetic diversity among African strains.

MLST, which has now been developed for more than 50 microbial taxa [24], revealed extremely low levels of polymorphisms in several protein coding genes of African *M. ulcerans* strains [16,17]. Analyses of the population structure of bacterial pathogens such as *M. tuberculosis* [25], *Yersinia pestis* [26] or *Salmonella enterica Typhi* [27]

have shown that single nucleotide polymorphism (SNP) typing is the most suitable fine-typing method for genetically monomorphic species [24]. This prompted us to develop a SNP typing method for *M. ulcerans* strains from a BU endemic area of Ghana. We selected two Ghanaian patient isolates representing two different Ghanaian VNTR types [19] for genome re-sequencing and compared obtained sequences with the published genome sequence of the reference strain Agy99 [28]. Whole genome comparison between these three strains detected 173 SNPs in total [29], which were used for the establishment of amplification refractory mutation system (ARMS) real-time PCRs using hairpin-shaped primers [30]. Typing of 74 strains isolated from patients living in the BU endemic Densu river basin at 65 SNP loci revealed the presence of five haplotypes in addition to the Agy99 reference haplotype. Sequencing of 4 additional strains chosen on the basis of detected haplotypes enabled further differentiation of isolates. Location of the homes of patients from whom the strains were isolated facilitated a phylogeographic analysis of haplotype distribution.

Materials and Methods

Ethics statement

In the present study, *M. ulcerans* isolates were obtained from BU diagnostic samples. Data were analyzed anonymously and bacterial isolates delinked from the patients from whom they originated. Ethical approval to use the diagnostic specimens for immunological and microbiological research was obtained from the ethical review board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. Written informed consent was provided by all patients for standard surgical treatment and anesthesia. In addition the ethical review board requested written informed consent for taking blood samples for immunological research on a special consent form, but not for potential later investigations of bacterial isolates generated during standard diagnostic procedures.

Mycobacterial strains and genomic DNA extraction

A total of 74 *M. ulcerans* patient isolates from a BU endemic area located in the Ga West, Ga East and Akuapim South Districts were included in the SNP typing analysis. Patients were aged 2 - 75 years, while 71% of the patients were younger than 15 years. In addition, *M. ulcerans* patient isolates from the Ashanti Region (Amansie West District) of Ghana and from other West-African countries (Ivory Coast, Togo, Benin, Democratic Republic of Congo and Angola) were enclosed for further SNP typing analyses (Table 1). The complete Agy99 genome sequence published in 2007 [28] and re-sequenced genomes of isolates NM20/02, NM31/04 [29], NM14/01, NM43/02, NM49/02 and NM54/02 were used as reference sequences. The genomes of strains NM14/01, NM43/02, NM49/02 and NM54/02 were analyzed at Monash University using an Illumina GAIIx Genome Analyzer. A 100x coverage per genome was obtained on average. The Short Read Mapping Package (SHRiMP) software was used for aligning the genomic reads against the target Agy99 genome. For SNP identification the Nesoni software was used. Genomic DNA was isolated by cell wall disruption and phenol-chloroform extraction as described previously [31].

SNP typing

Real-time PCR hairpin primer (HP) assays [30] were used to detect SNPs in the 74 Ghanaian *M. ulcerans* strains. Real-time PCR was performed using Power SYBR green 1x PCR Master Mix (Applied Biosystems), 5 ng genomic DNA and 0.3 µM forward and reverse primers each in a total volume of 25 µl. Reactions were carried out in a Step One Plus Real-time PCR system (Applied Biosystems) with a 96-well block. Thermal conditions were as follows and as described previously [30]: stage 1, 95°C for 10 min, 70°C for 30 s; stage 2, 72°C for 30 s, 95°C for 20 s, 69°C for 30 s, lowering one degree in the last step for every cycle during 10 cycles; stage 3, 72°C for 30 s, 95°C for 20 s, and 60°C for 30 s, repeated 40 times; Melt curve stage, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Data were collected in the last step of stage 3 and after the Melt curve stage for analysis with the Step One Software version 2.0 (Applied Biosystems). SNPs were detected by ARMS assays and for each assay two PCRs with two sets of PCR primers were performed in parallel. Each PCR reaction contains SNP-specific primers, which are designed to be either fully complementary to the DNA template or mismatched at the 3`end nucleotide. As reactions with totally complementary primers have a more rapid developing fluorescence curve and an earlier cycle-threshold, differences between the two reactions allow the detection of SNPs.

The hairpin-shaped primers were designed as described previously [30]. In the first step linear primers were designed with Primer3 [32] to produce short amplicons (30 to 90 bp) and to anneal between 60 and 65°C. A tail was added to the 5' end of the SNP-detecting primer in order to produce a stem with the 3' end of the primer. The stem was designed with mfold software (http://www.bioinfo.rpi.edu /applications/ mfold/old/dna/) to have a melting temperature of 67 to 70°C with a free energy of between -0.5 and -2.0. Primers are provided in Table S1.

Validation of SNP typing assays

Assays were validated on the published genome Agy99 as well as the reference strains NM20/02, NM31/04, NM14/01, NM43/02, NM49/02 and NM54/02 to confirm the presence of each allele and to verify the performance of SNP assays. Assays with genomic DNA samples from clinical isolates were considered reliable only if the cycle thresholds generated in the paired wells differed by three or more cycles and if the melting curves of paired wells were coherent. ARMS assays with low

discriminatory power were not included into the standard set of 65 SNP typing assays. Sanger DNA sequencing of PCR products was used to validate a selected subset of SNPs. Primers used for PCR and sequencing were designed using Primer3 [32] software. PCR was performed using FirePol 10x buffer and 0.5 µl FirePolTaq-Polymerase (Solis BioDyne), 5 ng genomic DNA, 0.72 µM forward and reverse primers each, 2mM $MgCl_2$ and 0.4 mM dNTPs (Sigma) in a total volume of 25 µl. PCR reactions were carried out in a Gene Amp PCR System 9700 PCR machine (Applied Biosystems). Thermal conditions for PCR amplification of *M. ulcerans* genomic DNA were as follows: initial denaturation step, 94°C for 5 min; 32 cycles: 94°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 1 min (elongation); final extension step, 72°C for 10 min. PCR products were analyzed on 1% agarose gels. PCR products were purified using the Nucleo Spin Extract II Kit (Macherey-Nagel). Sequencing of purified PCR products was done by Macrogen (World Meridian Venture Center, Seoul/Korea). Sequencing was conducted by the Sanger method using BigDye™ terminator cycling conditions using the Automatic Sequencer 3730xl (Applied Biosystems).

Several random SNP loci were validated by Sanger sequencing in order to verify the ARMS approach for a differentiation of *M. ulcerans* isolates. Additionally, validation of pivotal SNPs loci revealing different haplotypes within the endemic area around the Ga District as well as differences between Amansie West District strains and isolates from other African countries was carried out. 100% of 36 randomly chosen SNP loci tested in reference strains Agy99, Nm20/02 and Nm31/04 were reconfirmed by Sanger sequencing. 100% of 70 significant SNP loci dividing Ga District strains into 5 haplotypes other than Agy99 and distinguishing Amansie West District as well as African strains were likewise verified by Sanger sequencing. In contrast, four real-time PCR typing results, which were validated because of unique allele occurrences in certain Ga District isolates diverged from reconfirmatory Sanger sequencing analysis. Subsequent repetition of real-time PCRs in duplicates revised the initial real-time PCR analyses and confirmed Sanger sequencing results.

Phylogenetic analysis

MEGA software version 4.1 (beta) [33] was used to reconstruct the neighbor-joining tree based on SNP typing data (Phylogeny Test and options: Bootstrap 1000 replicates; Gaps/Missing Data: Complete Deletion; Codon Positions:

1st+2nd+3rd+Noncoding; Model: Nucleotide, Number of differences; Substitutions to include: Transitions + Transversions; Pattern among lineages: Same = Homogeneous; Rates among sites: Uniform rates).

We created a map of West Africa by using the map creator tool of Epi Info version 3.5.1 in order to illustrate detected SNP patterns in different African countries.

Results

Development of ARMS SNP typing assays

Recently we have compared 454 and Illumina genome sequencing data of *M. ulcerans* patient isolates NM20/02 and NM31/04 originating from two different BU endemic areas of Ghana with the published genome sequence of the Ghanaian strain Agy99 [29]. Based on the identification of 173 SNPs we have developed medium-throughput ARMS-based real-time PCR SNP assays with hairpin-shaped (HP) primers. Initial ARMS assays were successful for 108 of 173 detected SNP loci at predefined optimal conditions. We were able to discriminate alleles under a single standard condition at 73/108 (67.6%) SNP loci. This is close to the success rate of 72.4% reported by Hazbon and Alland for their first round of HP-assay design [30]. Real-time PCR analysis revealed sequencing errors in the Agy99 reference sequence at eight of these loci, which were thus not suitable for further typing analyses. Redesign of the 100 initially failed assays and ongoing whole-genome sequencing of additional isolates will increase the pool of discriminatory SNP assays for future analyses of the population structure of African *M. ulcerans*. Developed SNP assays were used to type a collection of strains from two different BU endemic areas of Ghana and other African countries (Figure 1).

Different clonal complexes of *M. ulcerans* dominate in the BU endemic regions of Africa

SNP typing of three strains from the Amansie West District of Ghana including the sequenced Amansie West reference strain NM31/04 revealed differences at 24 of the 65 SNP loci analyzed (37%) between these isolates (Figure 1B). In comparison to Agy99 differences at 29, 27 and 5 loci were found. A neighbor-joining tree analysis sub-grouped haplotypes from the two different BU endemic areas of Ghana into two clades (Figure 2). Clade 1 comprises strains isolated between 2001 and 2007 in the Densu river basin, which could be differentiated into 6 haplotypes (Figure 1A). All Amansie West District isolates are sub-grouped together with the 1999 isolate Agy99 into clade 2 (Figure 2).

Typing of patient isolates from other BU endemic African countries at the 65 SNP loci yielded 2 of 15 strains with patterns similar to haplotypes found in Ghana (Figure 1A and B). The only strain available from Togo had a haplotype which differed at only

one locus from haplotype 6 found in the Densu river basin. One of the two analyzed strains from the Ivory Coast had a haplotype similar to the haplotypes of strains from the Amansie West District of Ghana. The other isolate from the Ivory Coast as well as all seven analyzed strains from Benin, three strains from the Democratic Republic of Congo (DRC) and two strains from Angola shared a distinct SNP pattern when compared to the Ghanaian isolates (Figure 1A and B). Thus, SNP typing results of clinical isolates from Ghana and isolates from other African countries revealed a neighbor-joining tree with 3 main branches. The strain from Togo is sub-grouped together with haplotypes 2-6 into clade 1, while strain 1 from the Ivory Coast is classed with Amansie West District isolates and Agy99 into clade 2. The other 13 West-African strains are sub-grouped into clade 3 (Figure 2).

SNP typing of *M. ulcerans* patient isolates from a BU endemic area of Ghana identifies ten haplotypes belonging to a dominating clonal complex

Using the 65 established ARMS assays we SNP-typed 74 *M. ulcerans* patient isolates collected between 2001 and 2007 from the BU endemic Densu river basin of Ghana, from which the sequenced strains Agy99 and NM20/02 originated. Within this group of 74 strains of common geographical origin, differences at 14 of the 65 SNP loci tested (22%) were observed (Figure 1A). Altogether five haplotypes (designated haplotypes 2-6) other than the Agy99 associated haplotype 1 could be distinguished. Haplotypes 2 - 6 differed at 41, 43, 47, 48 and 55 of the 65 analyzed SNP loci from haplotype 1 (strain Agy99), respectively.

Based on detected haplotypes we re-sequenced 4 representative strains NM14/01 (haplotype 5), NM43/02 (haplotype 3), NM49/02 (haplotype 6) and NM54/02 (haplotype 4) in order to further differentiate strains from the same haplotype. We detected new unique SNPs in NM43/02 (26 SNPs), NM54/02 (11 SNPs), NM14/01 (9 SNPs) and NM49/02 (4 SNPs) and established 24 new assays, which enabled a segregation of each haplotype into two haplotypes (Figure S1). A phylogenetic tree for haplotypes 1-10 is shown in Figure 3.

Temporal and spatial distribution of haplotypes

In contrast to the generally rarer haplotypes 2-4; 6-8 and 10, haplotypes 5 and 9 were found within each time interval (one year) from 06/2001 to 06/2006 (data not shown). Haplotypes 1 and 2 were not found again in the whole strain collection.

Possible explanations include the actual absence of these haplotypes from the residual clonal *M. ulcerans* complex in the Densu river basin as well as phylogenetic or sampling bias.

For a phylogeographic analysis the homes of patients from whom the strains were isolated were marked in a map, depicting the distribution of haplotypes (Figure 4). Haplotypes 4, 6, 7, 9 and 10 appear to be unevenly distributed; i.e. they were found only in certain parts of the BU endemic area. Haplotype 10 was even found only within one small village. In contrast, the most prevalent haplotype (haplotype 5) co-localized with all other haplotypes. Interestingly, two *M. ulcerans* isolates of identical haplotype (haplotype 4) were isolated from two patients coming from the same household, suggesting a common source of infection.

Discussion

Previous investigations on the genetic diversity of *M. ulcerans* by comparative genomic hybridization analysis enabled differentiation of a world-wide collection of strains into two main lineages and six continental haplotypes [34]. However, phylogeographic and transmission pathway analysis requires high-resolution fine typing of strains from the same BU endemic region. This has been accomplished so far only for a few *M. ulcerans* populations and at low resolution. VNTR analysis of a Ghanaian strain collection based on the polymorphic loci ST1 and MIRU1 revealed the presence of three distinct allele combinations (BD/B, C/BAA and BD/BAA) in Ghana. All isolates from the Buruli ulcer endemic Densu river basin tested, except for Agy99, displayed combination BD/B [19]. Our recent genome re-sequencing analysis [29] compared the two Ghanaian VNTR type reference strains NM20/02 (BD/B) and NM31/04 (C/BAA) to Agy99 (BD/BAA) with the goal of detecting single nucleotide polymorphisms suitable for development of a fine-typing method. By selection of isolates with the three prevalent VNTR types in Ghana we expected to capture as much of the genetic variation present in the Ghanaian *M. ulcerans* population as possible. Our assumption was reinforced by re-sequencing four *M. ulcerans* strains initially grouped into haplotypes 3-6. Only 16 additional SNPs could be detected by comparison of the four strains to reference strain Agy99, while 50 unique SNPs could be identified by comparing the four strains among themselves. We anticipated that detected SNPs will provide the first useful genetic markers for phylogeographic and transmission pathway analyses at least in the Densu river basin and other BU endemic areas of Ghana.

We have identified 10 different haplotypes in a relatively small BU endemic area within the Densu river basin. Haplotypes 1-10 are descendants of a founder haplotype that has spread over the district; the most common haplotype 5 may represent this founder haplotype. Analysis of this spatial distribution of haplotypes indicates that emerging new haplotypes do not readily spread over the entire endemic area, but form focal transmission clusters.

Our data are comparable to studies of other genetically monomorphic organisms like *Salmonella typhi*, which report multiple strain types circulating within a specific

location [35-37]. Comparison of typing results in strains from two geographically separate BU endemic areas in the Densu river basin and the Amansie West Districts of Ghana uncovered a total of 61 differing alleles at 65 SNP loci. Within each of the two endemic areas SNP variation was significantly smaller (14 and 24 differences, respectively) than the overall variation between the two endemic areas. These results indicate the dominance of two different clonal complexes in the two separate Ghanaian BU endemic areas. SNP typing of two strains from Ghanaian neighboring countries showed similar SNP patterns when compared to AW District isolates (strain 1 from the Ivory Coast) or Ga District haplotypes (strain from Togo). Typing of 13 strains collected in additional African countries (Benin, DRC, Angola, strain 2 from the Ivory Coast) revealed a completely new SNP pattern compared to all other isolates. On the basis of typing with the Ghanaian set of SNPs these 13 strains could not be distinguished among each other and were thus clustered together into a clade. This clustering may however represent a phylogenetic discovery bias, i.e grouping of actually diverse strains leading to a so called “branch collapse”. Future addition of SNP loci identified by genome re-sequencing of a comprehensive pan-African selection of *M. ulcerans* isolates will lead to a further subdivision and differentiation of African strains. Phylogenetic discovery bias is implicit to SNP typing and will continue to exist as long as not every single sample will be sequenced. It will become smaller though with every additional re-sequenced genome. SNP typing based on a wider range of SNPs may therefore yield evidence for genetic divergence of strains from Benin, DRC, and strain two from the Ivory Coast. Hence, phylogeographic analyses in other African BU endemic areas will require whole genome comparison of strains from that area to develop a local set of informative SNPs. This is supported by our recent identification of seven insertion sequence element-related SNP types within Africa [38].

M. ulcerans has evolved from the aquatic environmental *M. marinum* and seems to have adapted to a more stable ecological niche [28]. Gain of the immunosuppressive toxin mycolactone is accompanied by loss of highly immunogenic proteins [39], suggesting an adaptation to survival in host environments that are screened by immunological defense mechanisms. Serological analyses have indicated that many individuals living in BU endemic areas are exposed to *M. ulcerans,* whereas relatively few develop clinical disease [9]. There is no published evidence for direct person to

person transmission of *M. ulcerans*. Based on numerous reports demonstrating an association of BU with slow flowing or stagnant water bodies, it is therefore commonly assumed that infection takes place through trauma of the skin or insect bites via an environmental reservoir in the aquatic ecosystems. Both biotic components, such as biofilms and aquatic invertebrate species are being considered as potential vectors and/or reservoirs [5,6,40-42]. Recent findings in south-eastern Australia have implicated mammals as environmental reservoir (Fyfe et al., submitted) and mosquitoes as vectors of *M. ulcerans* [43,44]. While large numbers of possums in a BU-endemic area of Australia are infected with *M. ulcerans*, a similar mammalian reservoir has not been identified in BU endemic regions of Africa. If such a reservoir plays a role in transmission, spread of *M. ulcerans* from chronic, ulcerated lesions to insect vectors or another currently unknown environmental reservoir should be considered. Subsequent infection of individuals living in the same settlements may be responsible for the focal transmission patterns, such as of haplotype 10, which was found only within one small village.

The Densu river basin represents one of the coastal drainage systems of Ghana. Its water is collected in the Weija Lake, which developed after construction of a dam at the river's mouth in the 1970s. If *M. ulcerans* bacteria would be carried freely by the flow of water of the Densu river and its tributaries, haplotypes present in upper parts of the river system should be represented in the lower parts. However, among patient isolates coming from the lower part of the basin close to the Weija Lake the rare haplotypes 4 and 7 dominated. *M. ulcerans* bacteria that are swept downstream in the river water thus do not seem to play a major role in transmission.

Results of this retrospective pilot study indicate that future longitudinal micro-epidemiological studies involving SNP typing of isolates may give deeper insight into transmission pathways and relevant reservoirs of *M. ulcerans.*

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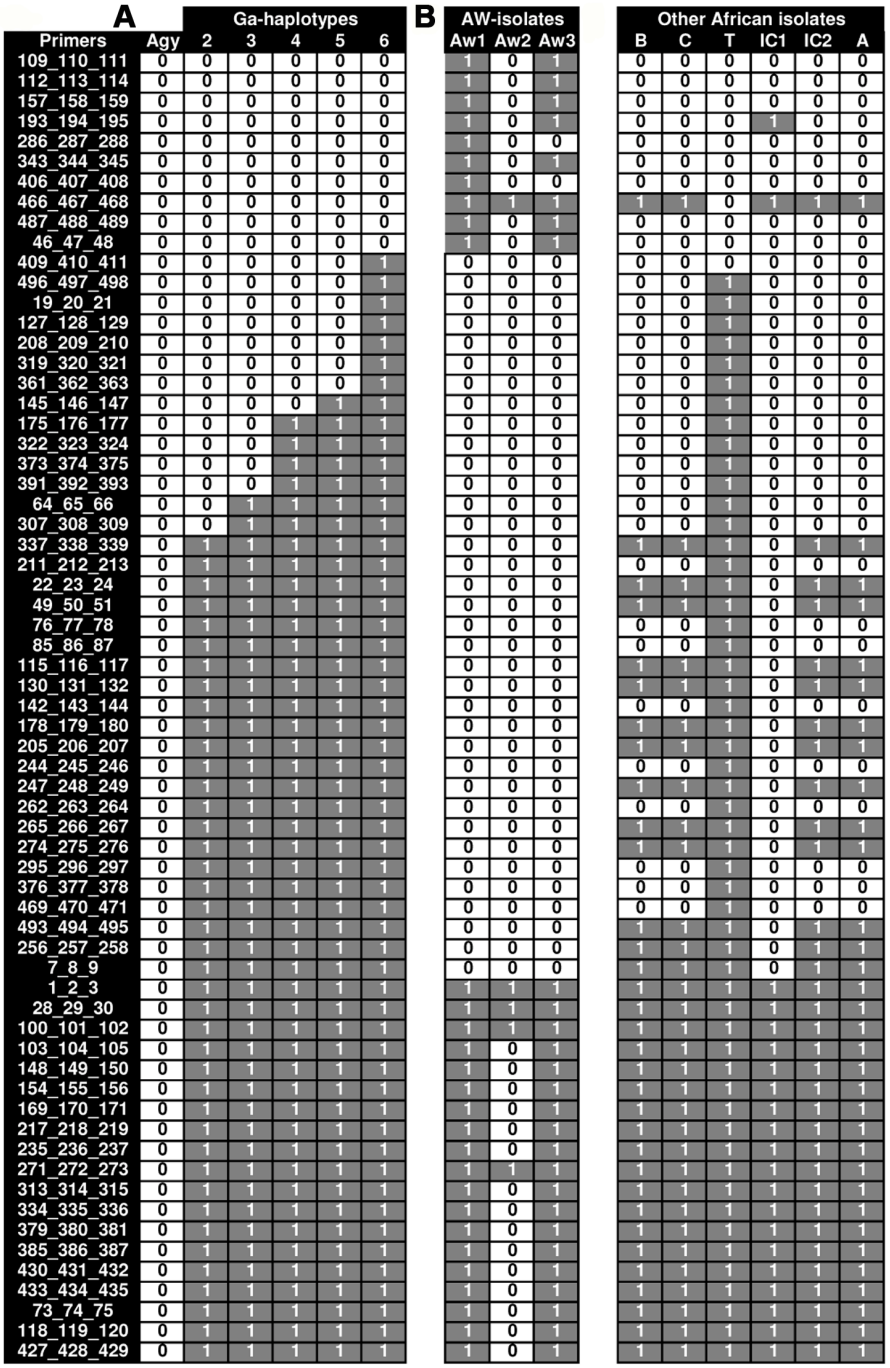
Table

Table 1. *M. ulcerans* strains included in the analysis

Number of strains	Year of isolation	Place of origin	VNTR type[1]
1 (Agy99)	1999	Ga District, Ghana	BD/BAA
38 (NM20/02)	2002	Ga District, Ghana	BD/B
21	2003	Ga District, Ghana	BD/B
5	2004	Ga District, Ghana	n.t.[2]
8	2005	Ga District, Ghana	n.t.
1	2006	Ga District, Ghana	n.t.
1	2007	Ga District, Ghana	n.t.
3 (NM31/04)	2004	AW District, Ghana	C/BAA
7	1997–2001	Benin	n.t.
3	n.p.[3]	DRC	n.t.
1	1997	Togo	n.t.
2	1994	Ivory Coast	n.t.
2	1996	Angola	n.t.

[1]ST1/MIRU1 allele [19].
[2]not tested.
[3]not provided.

Figures



A		Ga-haplotypes					B	AW-isolates			Other African isolates						
Primers	Agy	2	3	4	5	6		Aw1	Aw2	Aw3	B	C	T	IC1	IC2	A
109_110_111	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
112_113_114	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
157_158_159	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
193_194_195	0	0	0	0	0	0		1	0	1	0	0	0	1	0	0
286_287_288	0	0	0	0	0	0		1	0	0	0	0	0	0	0	0
343_344_345	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
406_407_408	0	0	0	0	0	0		1	0	0	0	0	0	0	0	0
466_467_468	0	0	0	0	0	0		1	1	1	1	1	0	1	1	1
487_488_489	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
46_47_48	0	0	0	0	0	0		1	0	1	0	0	0	0	0	0
409_410_411	0	0	0	0	0	1		0	0	0	0	0	0	0	0	0
496_497_498	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
19_20_21	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
127_128_129	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
208_209_210	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
319_320_321	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
361_362_363	0	0	0	0	0	1		0	0	0	0	0	1	0	0	0
145_146_147	0	0	0	0	1	1		0	0	0	0	0	1	0	0	0
175_176_177	0	0	0	1	1	1		0	0	0	0	0	1	0	0	0
322_323_324	0	0	0	1	1	1		0	0	0	0	0	1	0	0	0
373_374_375	0	0	0	1	1	1		0	0	0	0	0	1	0	0	0
391_392_393	0	0	0	1	1	1		0	0	0	0	0	1	0	0	0
64_65_66	0	0	1	1	1	1		0	0	0	0	0	1	0	0	0
307_308_309	0	0	1	1	1	1		0	0	0	0	0	1	0	0	0
337_338_339	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
211_212_213	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
22_23_24	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
49_50_51	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
76_77_78	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
85_86_87	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
115_116_117	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
130_131_132	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
142_143_144	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
178_179_180	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
205_206_207	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
244_245_246	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
247_248_249	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
262_263_264	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
265_266_267	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
274_275_276	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
295_296_297	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
376_377_378	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
469_470_471	0	1	1	1	1	1		0	0	0	0	0	1	0	0	0
493_494_495	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
256_257_258	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
7_8_9	0	1	1	1	1	1		0	0	0	1	1	1	0	1	1
1_2_3	0	1	1	1	1	1		1	1	1	1	1	1	1	1	1
28_29_30	0	1	1	1	1	1		1	1	1	1	1	1	1	1	1
100_101_102	0	1	1	1	1	1		1	1	1	1	1	1	1	1	1
103_104_105	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
148_149_150	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
154_155_156	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
169_170_171	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
217_218_219	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
235_236_237	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
271_272_273	0	1	1	1	1	1		1	1	1	1	1	1	1	1	1
313_314_315	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
334_335_336	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
379_380_381	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
385_386_387	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
430_431_432	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
433_434_435	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
73_74_75	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
118_119_120	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1
427_428_429	0	1	1	1	1	1		1	0	1	1	1	1	1	1	1

Figure 1. SNP typing analysis of African *M. ulcerans* isolates.

A *M. ulcerans* isolates from the Densu river basin of Ghana were analyzed at 65 SNP loci (Primer IDs) by real-time PCRs. Base exchanges relative to the reference sequence of strain Agy99 were registered as 1 (grey). Allele matches with Agy99 were recorded as 0 (white). 5 haplotypes in addition to haplotype 1 (Agy99) could be

distinguished on the basis of 14 SNP loci. **B** SNP typing results of strains from a second BU endemic area of Ghana as well as from additional African countries carried out with the set of SNP assays developed by whole genome sequencing of Ghanaian isolates. AW: Amansie West; Ga: strains from the Densu river basin; IC: Ivory Coast; T: Togo; B: Benin; C: Democratic Republic of Congo; A: Angola.

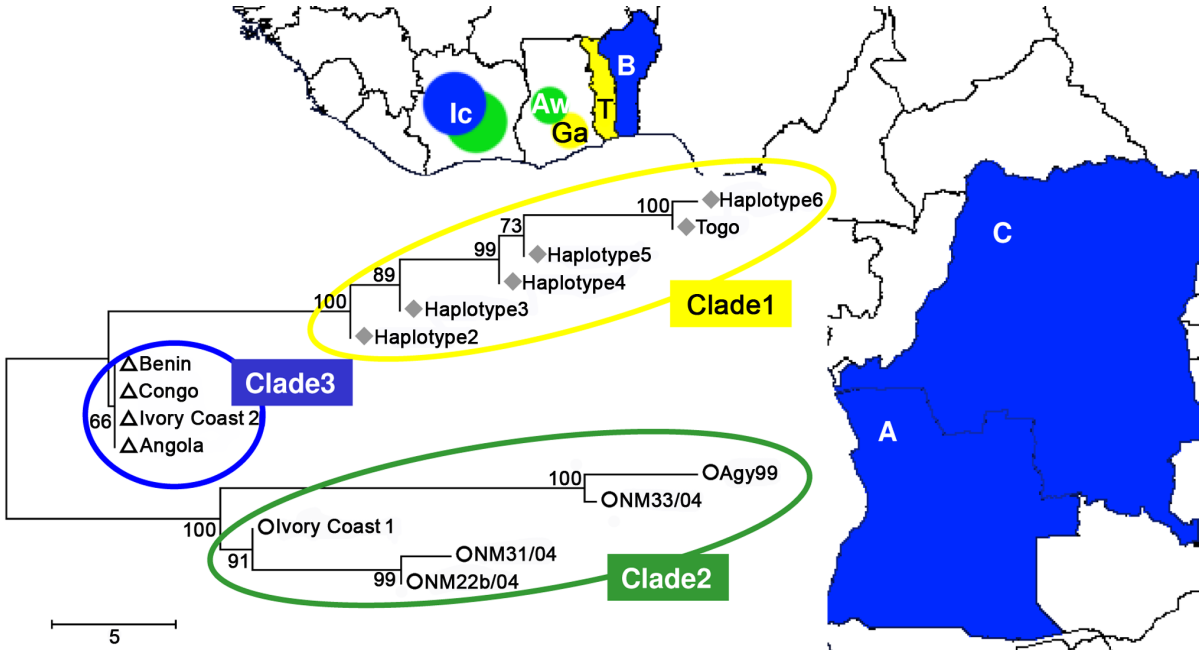


Figure 2. Geographical distribution of African *M. ulcerans* clades.

Map of West-Africa, showing the distribution and SNP haplotypes of three African *M. ulcerans* clades. Clade 1: yellow; clade 2: green; clade 3: blue. AW: Amansie West; Ga: strains from the Densu river basin; IC: Ivory Coast; T: Togo; B: Benin; C: Democratic Republic of Congo; A: Angola. A neighbor-joining tree shows sub-grouping of detected haplotypes from the Densu river basin together with the only strain from Togo into clade 1, strains from AW together with strain Agy99 and strain 1 from the Ivory Coast into clade 2 and all other strains from additional African countries into clade 3 (scale: number of differences at the SNP loci tested).

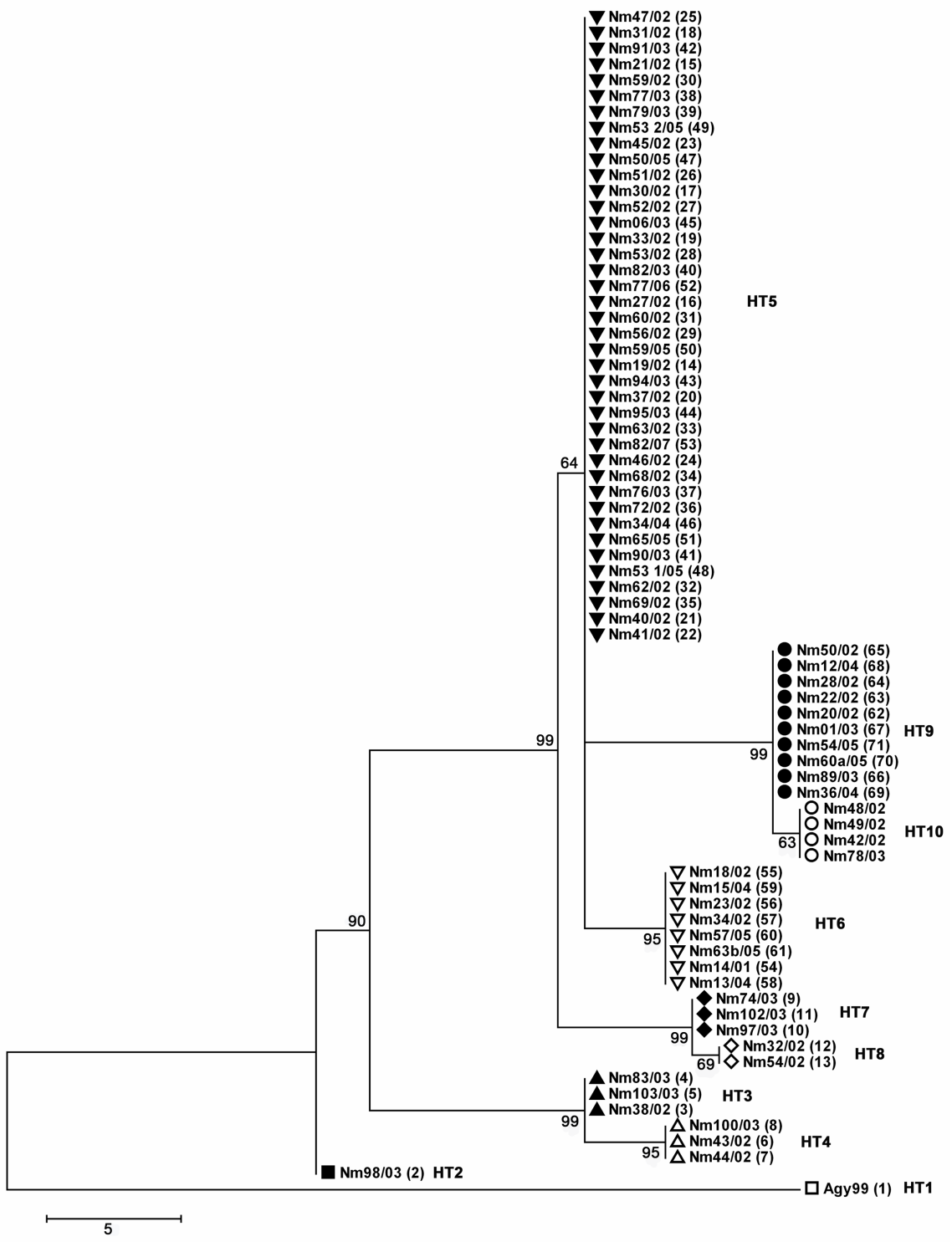


Figure 3. Neighbor-joining tree of 75 Ghanaian *M. ulcerans* isolates.

75 *M. ulcerans* isolates were aligned based on their SNP type (scale: number of differences at the SNP loci tested). HT = haplotype.

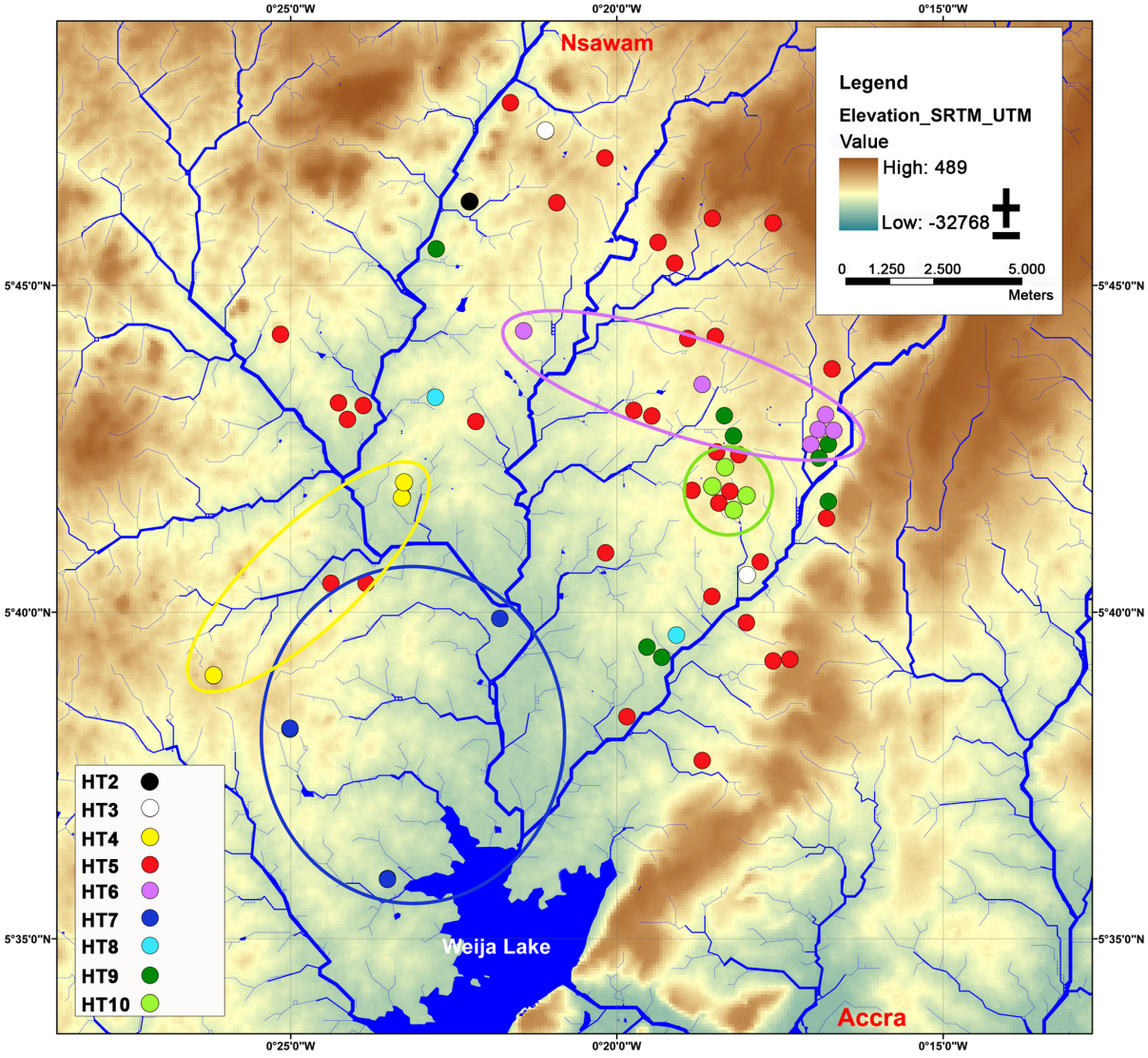


Figure 4. Geographical distribution of *M. ulcerans* haplotypes.

Map of the Densu river basin, showing the homes of patients from whom the strains have been isolated between 2001 and 2006 (colored dots). Haplotypes 2 (black), 3 (white), 4 (yellow), 6 (purple), 7 (dark blue), 8 (light blue), 9 (dark green), 10 (light green) are unevenly distributed, whereas haplotype 5 (red) co-localizes with all other haplotypes. The background map was created using elevation data from the Shuttle Radar Topography Mission (SRTM). Water bodies were classified using optical data from Landsat ETM and radar data from TerraSAR-XReferences.

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Chapter 10

General Discussion and Conclusions

10.1 General remarks

BU was first detected in Uganda 1897, but only after the first international Conference on “BU Control and Research” in 1998 in Yamoussoukro the disease gained significant public attention. BU causes, particularly in poor West African countries a substantial public health burden. It is assumed that all neglected tropical diseases (NTDs) together produce a burden of disease that may be equivalent to up to one-half of the sub-Saharan’s malaria disease burden and more than double the one caused by tuberculosis in Africa [1,2]. Since the Yamoussoukro conference many research efforts were undertaken and important insights into the nature of BU have been gained. However there are still many unsolved questions and problems especially in the field of transmission, diagnosis and treatment.

Improved case detection systems, a greater awareness of the disease in the population of endemic countries and the availability of an effective therapy might have contributed to the fact that case rates in some BU endemic areas seem to decrease. However, WHO estimates that still every year more than 5000 people worldwide are newly diagnosed with BU and the disease stays a major health problem [3,4].

Within the framework of the present thesis, histopathological analysis of tissue samples from BU patients and from experimentally infected mice allowed us to gain new insights into the pathogenesis of the disease, histopathological changes during treatment and causes for paradoxical reactions during and after chemotherapy.

10.2 Diagnosis of Buruli ulcer and monitoring of treatment success

In remote rural areas, diagnosis of BU is mostly based on clinical grounds although the disease can be confused with other skin diseases [5,6]. It is known that experienced health care physicians can reliably recognize, in particular advanced, BU lesions.
Nevertheless, a fast and easy laboratory diagnostic test would be very helpful for the correct diagnosis of pre-ulcerative lesions. Several methods for laboratory confirmation are currently available, of which most are only feasible in sophisticated labs. The only laboratory method suitable for rural health centers is microscopy, analyzing exudate smears of ulcerative lesions or fine-needle aspirations (FNA) of pre-ulcerative lesions for the presence of AFB [7-9]. However the sensitivity of this method is low, recognizing less then 50% of the BU cases [6]. Reference laboratories which are capable of performing other tests, like histopathology, cultivation of samples or IS2404 PCR confirmation are rare in the BU endemic African countries[10].
Cultivation is a very time-consuming technique often revealing false negative results because cultures are overgrown by other fast growing organisms or viable bacteria are absent in swab exudates. However, for genomic analysis (Chapter 7, Chapter 9) or phenotypic resistance testing cultivated bacteria are required. PCR detection of *M. ulcerans* DNA has a very high sensitivity, but is in turn prone for contamination and expensive lab equipment is needed [10,11]. Diagnosis by histopathological examination can be reliable if tissue specimens are taken carefully from the right place and analyzed by a trained person (Chapter 4, Chapter 5) [6,12-14].
To monitor treatment success all these methods do not allow to differentiate between viable and dead bacteria. In our mouse study we could still observe AFB 12 weeks after start of chemotherapy in the histopathological samples, although CFU counts were already negative for a long time. Furthermore human BU lesions remain IS2404 PCR positive for a long time after completion of antimycobacterial treatment (unpublished results). Detection of AFB or DNA thus cannot be used to determine the activity of the infection. Currently, the most reliable method to determine viability of the *M. ulcerans* bacteria is in vitro cultivation. New methods to overcome this lack are under evaluation. One possibility would be the detection of mycolactone in tissue. It is assumed that the level of mycolactone is declining very fast after killing of the bacteria and therefore this parameter would probably represent a good surrogate marker to determine viability and to monitor the efficacy of antibiotic or heat therapy.

Currently several groups are working on the development of a mass spectrometry method to detect and quantify mycolactone in tissue and wound exudates. It has been shown that it is possible to extract detectable amounts of mycolactone from tissue and from circulating blood monocytes [15].

The thin-layer chromatography (TLC) method was improved and adapted by Kishi and Spangenberg to detect mycolactone in the acetone soluble lipid fraction. Although this method is cheap and easy to handle sensitivity has to be improved in order to directly detect mycolactone in human tissue [16,17]. Another option, independent of mycolactone, could be the detection of mycobacterial RNA inside tissue samples as it is used in leprosy and tuberculosis [18,19]. For RNA detection as well as mass spectrometry expensive lab equipment is needed making it impossible to perform these assays in remote rural areas. A modification of the Quantigene RNA ELISA method could represent a format that is adaptable to the field.

Another approach for the development of a point-of-care diagnostic test could be the development of an easy to handle serological test format, such as a dip-stick, which can be used on spot and gives a fast and reliable result [20,21]. First antigens were already evaluated, in particular the 18KD small heat-shock protein from *M. ulcerans*. Unfortunately, not only patients but also healthy household contacts and people living in endemic areas showed reactivity [22]. Another group used *M. ulcerans* specific proteins identified by comparative genomics which are useful to determine exposure to *M. ulcerans* and for sero-epidemilogical studies [23].

During our histopathological analysis of mouse and human tissue samples infected with *M. ulcerans* (Chapter 3, Chapter 4, Chapter 5), we observed bacteria with an incomplete ZN staining, the so-called beading. This phenomenon was discovered in *M.leprae* and is due to a decomposition of the mycobacterial cell wall [24,25]. In *M. ulcerans* infected mice we observed already one week after commencement of antibiotic treatment a change of the ZN staining and the occurrence of beading (Chapter 3). Beading correlated with the inability to cultivate the bacteria. At close examination, the same phenomenon was observed in tissue from treated BU patients (Chapter 4, Chapter 5). This led us to the assumption that also in BU beading of bacteria is a marker for non - viability and can be used to estimate treatment success or the probability of relapse.

10.3 Treatment of BU

Until 2004, the only recommended treatment for *M. ulcerans* was the extensive surgical excision of the ulcerated tissue down to the deep fascia and into the healthy margins of the lesion, followed by skin grafting. In a high percentage of patients the removal of mycobacteria was incomplete and recurrences appeared in up to 47% of the cases [26-29]. Tissue that appeared macroscopically healthy frequently harbored *M. ulcerans* bacteria able to form new infection foci [30].

Studies with antibiotics in mice showed no benefit until the combination of rifampicin and amikacin was used and showed bactericidal effectivity against *M. ulcerans* [31-34]. Following these results, a small clinical trial with pre-ulcerative lesions in Ghana using the combination of rifampicin and streptomycin, reported efficacy of this combination and led in 2004 to the provisional WHO recommendations to treat BU patients with a combination of rifampicin and streptomycin for 8 weeks [35-37]. Although this treatment is effective and recurrence rates are below 2%, it is associated with several problems [37,38]. First of all, rifampicin is a frontline drug in the treatment of *M. tuberculosis* and the 8 weeks treatment for BU may be associated with the risk to develop resistant tuberculosis strains in patients with a BU/TB co infection. Beside this there are several other complications linked to the antibiotic treatment. O'Brien reported for the first time the occurrence of paradoxical reactions in BU patients receiving antibiotic therapy [39]. These reactions have similarities to the immune reconstitution inflammatory syndrome (IRIS) in HIV/*M .tuberculosis* co-infected patients who commence anti-retroviral therapy [40-42].

A worsening of the wound during treatment is frequently observed [37,43] leading to the question whether a prolonged antibiotic therapy or a change in the treatment regimen is necessary. Careful retrospective evaluation revealed that worsening of the lesion was not due to a treatment failure but rather a strong immune response towards mycobacterial antigens still present inside the tissue [39,44].

During our studies we examined two patients who developed secondary lesions (Chapter 5) during or after successful initial antibiotic treatment at various body sites, at some distance to the initial lesion. Secondary lesions should not be confused with untreated patients presenting before commencement of treatment with multiple lesions, the so called metastatic, BU disease [45]. In earlier times, secondary lesions were thought to be due to a new infection or relapse and additional treatment would

be necessary. Our histopathological analysis identified secondary lesions as an inactive BU lesion with typical histopathological hallmarks observed after antibiotic therapy (granulomas, B-cell cluster) [46]. This suggests that no additional antibiotic therapy is necessary but minimal surgical excision might be beneficial.

Plaques are the most problematic pre-ulcerative stages to treat, due to the frequently observed occurrence of ulceration during antibiotic therapy. The results we obtained revealed large necrotic areas still present after completion of standard antibiotic therapy. No indications for treatment failure were observed in ulcerating lesions. However the immune system was not capable to clear large necrotic tissue areas entirely, leading to an opening of the wounds. Plaque patients which received surgical wound débridement showed a faster improvement and a shorter hospital stay. Wound cleaning and the transition from a chronic wound into an acute wound has been shown beneficial in several other diseases (venous ulcer, diabetic ulcer) and to favor the healing process [47-49]. This might not be only true for plaque lesions but for all larger BU wounds with a delayed and slow healing.

For clinical decision making it is thus important to differentiate immune-mediated paradoxical reactions from relapses or new infections.

The possibility to discriminate closely related strains by genetic fingerprinting (Chapter 9) may help to differentiate between re-infection and relapse [50].

A fully oral treatment would simplify chemotherapy and adverse side effects would be minimized if streptomycin could be omitted. A first clinical trial with 30 patients from Benin (Chapter 6) showed that treatment efficacy with the combination of rifampicin/clarithromycin is comparable to that with rifampicin/streptomycin and no differences in the histopathological changes during treatment could be observed [44,51].

Another promising treatment alternative is the thermotherapy which is based on the finding that *M. ulcerans* grows best at a temperature of 30°C and is killed at higher temperatures [52]. This property was already used in a first thermotherapy study in the seventies [53]. Recently Junghanss *et al* published a proof of principle trial in which a phase change material to produce heat was used and six BU patients were successfully cured [54]. Currently a large trial to evaluate safety, applicability and effectiveness of thermotherapy is ongoing and first results will be available 2012.

10.4 Immune response

Early histopathological examinations of BU lesions revealed a complete absence of infiltration and the presence of large necrotic areas. Destroyed fat tissue with the presence of fat cell ghosts and foci of extracellular bacterial clusters were identified as histopathological hallmarks of BU [55]. Our studies in mice showed that one week after inoculation a strong neutrophilic infiltration could be observed which later on changed to a more macrophage- dominated response followed by the formation of the typical necrotic areas (Chapter 3). Although human lesions of these early stages are not available for analysis we found in more advanced lesions neutrophilic debris to be present inside the necrotic core, most likely the remains of an early wave of infiltration (Chapter 5). The proportion of individuals that can clear a *M. ulcerans* infection before clinical signs and symptoms emerge is not known. However, serological studies with *M. ulcerans* antigens revealed a high number of sero-positive but healthy people in BU endemic areas [22].

After commencement of antibiotic therapy the histopathological appearance changes dramatically [46,56]. We observed a strong influx of immune cells into the lesion, especially the formation of a rim of macrophages around the necrotic areas (Chapter 5), the formation of granulomas as well as of B-cell cluster (Chapter 4, Chapter 5).

Whether HIV infection increases the risk to develop BU is not well investigated. Co-infections of BU/HIV have been reported with severe BU clinical presentation [57,58]. A case–control study comparing HIV-1/HIV-2 sero-prevalence in BU patients from Benin suggests that HIV sero-positivity increases the risk for BU. Another study showed that BU is more aggressive and osteomyelitis is more common in HIV positive patients [57,58] We are planning a systematic histopathological study with HIV/BU co-infected patients in order to characterize if immune and treatment responses are comparable to non-HIV patients. Due to the reduction in CD4 T- cell counts the immune response and the histopathological presentation might be altered compared to non-HIV patients.

10.5 Genetic fingerprinting and Transmission

The full genome of the Ghanaian *M. ulcerans* strain Agy99 was published in 2007 [59] by the group of Tim Stinear. Compared to *M. marinum*, *M. ulcerans* lost over 1.1 Mb of DNA caused by deletions and acquired the virulence plasmid pMU001. Comprised in the deleted genes were many virulence factors and immunodominant antigens compared with its progenitors *M. marinum* and *M. tuberculosis. EsxA* and E*sxB* are both important virulence factors in *M. tuberculosis* and we could show that both are deleted in strains belonging to the classical lineage of *M. ulcerans* (African and Australian strains). In strains of the ancestral lineage loss-of-function mutations and partial deletions were observed (Chapter 7). This suggests that the loss provides a selective advantage [59,60].

Due to the monomorphic population structure of *M. ulcerans,* belonging to the classical lineage, typing by conventional methods (MLST, VNTR) resulted only in a low resolution [61-63]. During our studies Ghanaian strains from the same geographical region were compared and a fine-typing method based on single-nucleotide polymorphisms (SNPs) was established [50] (Chapter 9). With this method it was for the first time possible to distinguish clinical isolates from one BU endemic region.

Dominance of one clonal complex and local clustering of some of the variants belonging to this complex was observed [50]. This method might help to answer some of the open questions concerning transmission. By genetic comparison of clinical isolates with environmental samples from soil, insects and water it might be possible to follow and elucidate the mode of transmission and the reservoir. Additionally, genetic fingerprinting should help to differentiate between relapse (same strain) and new infection (different strain) (Chapter 5). If more than one family member is suffering from BU a fine typing method would reveal if the same strain causes the disease in all members. Finally, in patients with multiple lesions one could investigate if all lesions are caused by the same strain which may have spread via lymphatics and blood to distant sites (Chapter 5). The drawback of this method is that for each region a unique set of SNPs has to be defined and evaluated. In the near future whole genome comparison of large strain collections will be the method of choice.

10.6 Vaccine development and disease control

Although an effective antibiotic therapy to cure BU is available, it is tedious, expensive, requires injections and treatment failures are observed. Prevention of the disease by avoiding risks for infection is very difficult because the mode of transmission is not clear and people may get in touch with reservoirs of the pathogen during their daily live while fishing, farming and washing [64,65]. Therefore, the availability of a vaccine for prevention would be highly desirable.

Whether a protective immune response against *M. ulcerans* can develop is so far not known but several indications support this hypothesis:

1. Serological studies revealed that household contacts show an immune response against mycobacterial antigens without signs of the disease [22]. Whether sero-positive individuals are protected against future infections has not been evaluated and it is unclear if this immune response was primed by *M. ulcerans* or by an infection with other cross- reactive environmental mycobacteria.
2. Limited BCG vaccination effectivity against *M. ulcerans* disease was shown in several trials with a protection for up to six month and reduced risk of developing severe BU form, including osteomyelitis [66-68]. Additionally, mouse studies with BCG showed a delayed onset of the disease in BCG vaccinated animals. However variable results were obtained when using different *M. ulcerans* isolates and different mouse strains [69].
3. Our in depth analysis of secondary lesions emerging after chemotherapy (Chapter 5) or thermotherapy (unpublished results) suggest that a priming of the immune system occurs during initial treatment, leading to the ability to resolve bacterial foci formed either by newly infecting bacteria or by spreading of bacteria which survived the initial treatment (Chapter 5).
4. Positive cultures after 8 weeks of RIF/STR treatment were observed in several clinical trials as well as in our mouse study (Chapter 3). Surprisingly, lesions resolved without prolongation of the antibiotic therapy indicating development of an immune response to *M. ulcerans* which was sufficient to contain the infection[70].

The polyketide toxin mycolactone would be an obvious candidate for a vaccine but no natural immune response against it has been observed. Carrier conjugates of this macrolide showed no strong immunogenicity in mice [71] and so far only one

monoclonal antibody against mycolactone was generated (JP Dangy, unpublished results).

Research efforts have recently been started (BuruliVac Project) to develop either a live attenuated or a subunit vaccine against *M. ulce*rans. Subunit vaccines, based on recombinant proteins or plasmid DNA represent a safe strategy in contrast to live attenuated vaccines. For protein subunit vaccines protective antigens as well as a suitable adjuvant system are required. For pre-clinical development a mouse infection model is available.

Irrespective of these research efforts it is extremely important to strengthen access to health care in the affected remote communities. Additionally, implementation of surveillance activities and sensibilization of the population to recognize BU at a very early stage are needed in order to reduce the morbidity and sequelae of the disease. Access to physiotherapy for the prevention of disability and rehabilitation is another important element [3].

In many rural areas people still believe in witchcraft as the cause of the disease and prefer to seek care at traditional healers which delays start of antibiotic treatment. Cost of treatment, duration of admission and the distance between the hospital and the homes of patients also influence the health seeking behaviour [72]. To overcome these problems patients should be aware of the symptoms, know what causes the disease and their confidence in a successful and helpful treatment should be strengthened. A decentralisation of health care and regular visits of health workers to remote rural areas may help to fulfil these goals and this will hopefully lead to an earlier diagnosis and treatment in most of the BU patients.

10.7 Conclusions

In this thesis the histopathological analysis and evaluation of *M. ulcerans* infected tissue samples is described. The results contribute to our understanding of the pathology and treatment associated changes of *M. ulcerans*. The most important scientific findings are:

1. The BU mouse footpad model was used to study in detail the early pathogenesis and the effects of antibiotic treatment. Histopathology revealed that early infiltrate, mainly composed of neutrophils and macrophages emerge early after inoculation. In contrast B-cell cluster and T-cells are found much later in the course of chemotherapy. Granuloma formation as usually observed in antibiotic- treated human patients was not seen in mice. Beading of AFB was found to a marker of loss of viability in *M. ulcerans*.

2. The histopathological analysis of plaque lesions, which tend to ulcerate during antibiotic treatment, revealed large necrotic areas still present after 8 weeks of standard antibiotic therapy. Limited surgical excision may lead to a faster healing and shorter hospital stays.

3. Analysis of secondary BU lesions, which occurred up to 14 month after completion of antibiotic treatment, revealed typical signs of inactive BU lesions. These lesions may be caused by an immune- driven paradoxical reaction against mycobacterial antigens released from bacilli killed by the primary chemotherapy. Alternatively they may be secondary infection foci controlled by immune responses, primed during initial treatment. Additional antibiotic treatment is obviously not necessary.

4. A purely oral treatment with a combination of rifampicin and clarithromycin showed a very good effectivity. Histopathological changes were comparable to these observed in RIF/STR treated patients.

5. Genetic analysis revealed that the two copies of the *esxB-esxA* gene cluster found in *M. marinum M* are deleted from the genome of *M. ulcerans* strains

belonging to the classical lineage. Loss of expression of highly immunogenic proteins is an indication that *M. ulcerans* is adapting to a more stable environment that is screened by an immune system.

6. A DNA extraction protocol for *M. ulcerans* was developed which greatly enhanced both the yield and the purity of isolated DNA.

7. The development of a real-time PCR method based on the analysis of SNPs (single-nucleotide-polymorphisms) represented a milestone in genetic typing of *M. ulcerans.*

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Curriculum Vitae

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Education

Since 04/ 07 **PhD thesis (Molecular Immunology/ Microbiology)**
Mycobacterium ulcerans disease and treatment: a histopathological perspective
Swiss Tropical and Public Health Institute,
University of Basel, Switzerland

10/04 - 03/06 **Master of Science in Infection Biology and Epidemiology** (Grade 6.0)
"Generation of monoclonal antibodies reactive with mycobacterial PPE proteins"
Swiss Tropical and Public Health Institute
University of Basel, Switzerland

10/01 - 10/04 **Bachelor of Science in Biology** (Grade 5.0)
Major in Integrative Biology
University of Basel, Switzerland

09/92 – 08/01 **High school** (Grade 2.1)
Markgräfler Gymnasium, Müllheim, Germany

Work experience

07/09 **Visiting researcher, TB unit** (qPCR of clinical and environmental samples)
Victorian Infectious Diseases Reference Laborotory, Melbourne, Australia

07/06 – 04/07 **Technician in cancer research, oncology/cell biology group**
Basilea Pharmaceutica AG, Basel

Presentations

11/04/11 **Talk** *(Mycobacterium ulcerans disease and treatment: a histopathological perspective)*
Swiss TPH, Research Seminar, Basel, Switzerland

28/03-30/03/11 **Talk** *(Skin lesions emerging several months after completion of antibiotic treatment of Buruli ulcer: evidence for immunity against Mycobacterium ulcerans?)*
Annual meeting of the Global Buruli ulcer Initiative,
World Health Organisation, Geneva, Switzerland

30/03-03/04/09 **Talk** (*Histopathological monitoring of the development of Buruli ulcer and the changes after Chemotherapy in the murine footpad model)*
Annual meeting of the Global Buruli ulcer Initiative,
World Health Organisation, Cotonou, Benin

22/10/08 **Talk** *(Histopathological monitoring of Buruli ulcer and the changes after chemotherapy in the murine footpad model)*
SSTMP PhD student meeting, Yongy, Switzerland

17/03 – 19/03/08 **Poster** *(Immunohistological analysis of the cellular immune response and bacterial load in M. ulcerans infected mice during chemotherapy)*
20th Meeting of the Swiss Immunology PhD students
Schloss Wolfsberg, Ermatingen, Switzerland

Publications

accepted **Ruf MT, Schütte D, Chauffour A, Ji B, Jarlier V, Pluschke G**
Chemotherapy associated changes of histopathological features of *Mycobacterium ulcerans* lesions in a Buruli ulcer mouse model
Antimicrobial Agents and Chemotherapy

2011 **Ruf MT, Sopoh GE, Brun LV, Dossou AD, Barogui YT, Johnson RC, Pluschke G**
Histopathological changes and clinical responses of Buruli Ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue?
PLoS Negl Trop Dis

2011 **Ruf MT, Chauty A, Adeye A, Ardant MF, Koussemou H, Johnson RC, Pluschke G**
Secondary Buruli Ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection?
PLoS Negl Trop Dis

2011 **Chauty A, Ardant MF, Marsllier L, Pluschke G, Landier J, Adeye A, Goundoté A, Cottin J, Ladikpo T, Ruf MT, Ji B**
Oral treatment for *Mycobacterium ulcerans* infection: results from a pilot study in Benin
Clin Infect Dis 2011 Jan;52(1):94-6.

2010 **Pidot SJ, Porter JL, Marsollier L, Chauty A, Migot-Nabias F, Badaut C, Bénard A, Ruf MT, Seemann T, Johnson PD, Davies JK, Jenkin GA, Pluschke G, Stinear TP**
Serological evaluation of *Mycobacterium ulcerans* antigens identified by comparative genomics
PLoS Negl Trop Dis. 2010 Nov 2;4(11):e872.

2010 **Röltgen K, Qi W, Ruf MT, Mensah-Quainoo E, Pidot SJ, Seemann T, Stinear TP, Käser M, Yeboah-Manu D, Pluschke G.**
Single nucleotide polymorphism typing of *Mycobacterium ulcerans* reveals focal transmission of buruli ulcer in a highly endemic region of Ghana.
PLoS Negl Trop Dis. 2010 Jul 20;4(7):e751.

2009 **Käser M, Ruf MT, Hauser J, Marsollier L, Pluschke G**
Optimized method for preparation of DNA from pathogenic and environmental mycobacteria
Appl Environ Microbiol. 2009 Jan;75(2):414-8

2008 **Huber CA, Ruf MT, Pluschke G, Käser M**
Independent loss of immunogenic proteins in *Mycobacterium ulcerans* suggests immune evasion
Clin Vaccine Immunol. 2008 Apr;15(4):598-606

Laboratory Skills

Histology	Standard techniques, immunohistochemistry,
Cell culture	Monoclonal antibody production, cultivation of adherent and suspension cell lines
Drug screening	Nuclear fragmentation assay, cell cycle analysis, FACs analysis,
Standard techniques	ELISA, Western, PCR, qPCR

Computer and Languages

Computer: Microsoft Office (Word, Exel, PowerPoint), Adobe Photoshop, Graphpad prism, basic knowledge of STATA 8

Language:
German: native speaker
English: fluent
French: basic knowledge